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(54) ENHANCER DNA BASE SEQUENCE OF MOULD AND ITS USE

(57)Abstract:
PURPOSE: To obtain the subject new base sequence having a specific base sequence, bond to Aspergillus oryzae-derived a-glucosidase gene promoter domain, and capable of efficiently producing useful proteins, peptides using mould as host.
CONSTITUTION: This new base sequence is a new enhancer DNA base sequence containing a sequence: -CGGNNATTTA-. This base sequence is incorporated into the promoter domain of an a-glucosidase gene derived from mould such as Aspergillus oryzae and binds a marker gene suitable for selecting the transformants of the mould as host, a terminator and a DNA domain replicable by Escherichia coli, and then incorporated into the host to enable useful proteins and peptides to be produced in high efficiency. This enhancer DNA base sequence is obtained by isolating the a-glucosidase gene promoter and by conducting a retrieval of this base sequence by using a gene-analyzing software.

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* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.

2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

CLAIMS

[Claim(s)]
[Claim 1] The enhancer DNA sequence containing array-CGGNNATTTA-.
[Claim 2] The enhancer DNA sequence according to claim 1 whose NN is GC.
[Claim 3] The enhancer DNA sequence containing array-CCAATCAGCGT-.
[Claim 4] The enhancer DNA sequence containing an array according to claim 1 or 3.
[Claim 5] The enhancer DNA sequence according to claim 4 whose NN is GC.
[Claim 6] The amelioration promotor characterized by introducing one or more enhancer DNA sequences according to claim 1 or 3 into the promoterregion which functions with mold.
[Claim 7] The amelioration promotor according to claim 6 whose promoterregion is the promoterregion of the hydrolase gene of the mold origin, or a glycolytic pathway enzyme gene.
[Claim 8] The amelioration promotor according to claim 6 whose promoterregion is the promoterregion of the alpha-glucosidase gene of the Aspergillus oryzae (Aspergillus oryzae) origin shown by array number:2, or promoterregion including the partial array.
[Claim 9] The plasmid for a polypeptide manifestation in mold which has an amelioration promotor according to claim 6, has a suitable marker gene for selection of the transformant of host mold, has a terminator, and has the DNA field which can be reproduced with Escherichia coli.
[Claim 10] The plasmid according to claim 9 whose marker gene is the nitrate reductase gene, the ornithine-carbamoyltransferase gene, tryptophan-synthase gene, or the aceto amidase gene of the mold origin.
[Claim 11] The plasmid according to claim 9 whose marker gene is a nitrate reductase gene of the Aspergillus origin.
[Claim 12] The plasmid according to claim 9 whose terminator is the terminator of the alpha-glucosidase gene of the Aspergillus oryzae origin shown by array number:3, or a terminator including the partial array.
[Claim 13] The plasmid according to claim 9 which has DNA which carries out the code of the polypeptide between a promotor and a terminator.
[Claim 14] The manufacturing method of the polypeptide which consists of introducing a plasmid according to claim 13 into mold, and cultivating the obtained transformant.

[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the useful protein using an effective new enhancer DNA sequence, the promotor who introduced this, the plasmid for a manifestation including the promotor, and it, and a peptide manufacturing method, in order to produce useful protein and a peptide for mold efficiently as a host.
[0002]
[Description of the Prior Art] As a host of the useful protein by transgenics, Escherichia coli and yeast have so far been used mainly. However, in Escherichia coli, when different-species protein is made to high-discover, insoluble protein is not formed in a fungus body, or posttranslational modification, such as addition of a sugar chain characteristic of the protein of eukaryote, does not happen, but in order to obtain protein with activity, there is a fault of being accompanied not a little by difficulty. Moreover, in yeast, although addition of a sugar chain took place, it had the problem that a volume was low.
[0003] On the other hand, mold carries out secretory production of various kinds of enzyme protein so much out of a fungus body. On the stock of the Aspergillus nigre used industrially, glucoamylase For example, 20g or more per 1l. of culture medium, Moreover, since Aspergillus oryzae is also said to produce about 50g per kg of alpha-amylases by solid culture and Trichoderma reesei which is a cellulase production bacillus also has the enzyme productivity of this level It is expected that congener, of course, hides the possibility of high secretory production also about different-species protein. Moreover, there is much what is used for manufacture of brewing food or production of the enzyme for food processing like Aspergillus oryzae or Aspergillus nigre, such mold is estimated to be safe as a host in the various organs concerned (FDA), for example, U.S. Food and Drug Administration, and it is thought that the useful protein production by such mold is also easy to be approved.
[0004] From such a point, mold has come to be reported to production of various protein as a host of gene engineering in recent years as Escherichia coli and a microorganism which replaces yeast. although the productive efficiency is regulated by various factors, such as disassembly of the protein by posttranslational modification, such as the amount of imprints by the promotor who uses it, translation effectiveness after an imprint, and sugar chain addition of the discovered protein, the copy number of a gene, and the host protease, when producing target protein by gene engineering, if there are not many amounts of imprints of purpose protein in the first place probably -- the manifestation secretion more than the amount of imprints -- it should also wish -- there is nothing. Since how a powerful promotor is acquired from this viewpoint poses a big problem, various promotors are isolated also in mold and production of purpose protein is reported using these. The

promotor [Biotechnology, 5, and 368 (1987)] of the glucoamylase gene of Aspergillus nigre, the promotor [Biotechnology, 6, and 1419 (1988)] of the alpha-amylase gene of Aspergillus oryzae, the promotor [Biotechnology, 7, and 596 (1989)] of the cello BIOHAIDORAZE gene of Trichoderma reesei, etc. are raised as the example of representation.

[0005] However, the present condition is that the research on the gene engineering of mold is behind compared with other microorganisms, and the gene expression device about a promotor is hardly solved yet, but the example of a report has been increasing at last in the past several years. For example, the research on the catabolite inhibitor [Mol.Microbiol., 7, and 847-857 (1993)] related to negative manifestation control of the gene about use of a carbon source or the joint factor [Mol.Gen.Genet., 235, and 81 (1992)] of a CCAAT array found out widely in the gene expression control region of eukaryote etc. is raised. Therefore, the attempt which depends for production of the useful protein by mold on a promotor's original manifestation control to be used, the present condition is choosing and using the promotor according to the purpose, improves a promotor's expression control force, and acquires a more useful promotor is not performed to current. Therefore, a promotor with more high usefulness not only can acquire, but without isolating a new high manifestation promotor, if a promotor's amelioration technique is offered, by improving the existing promotor, the promotor's manifestation control also becomes possible and the more efficient production of useful protein of it is attained.

[0006]

[Problem(s) to be Solved by the Invention] The purpose of this invention identifies, a promotor's analysis about manifestation control, for example, enhancer nucleic-acid base sequence, and is to solve the function of this enhancer sequence. Furthermore, a promotor is improved using this, the manifestation plasmid using this promotor is built, and it is in developing the protein manifestation system by carrying out the transformation of the mold using this plasmid.

[0007]

[Means for Solving the Problem] In order to attain the above-mentioned purpose, this invention persons succeeded in the exclusion of a dependency to increase and the carbon source of a promotor's transcriptional activity by finding out the new enhancer nucleic-acid base sequence deeply concerned with forward expression control, as a result of inquiring wholeheartedly, isolating the promotor of the alpha-glucosidase gene (agdA) of Aspergillus oryzae and performing this promotor's detailed analysis, and introducing two or more these enhancer sequences into a promotor. Furthermore, the new manifestation plasmid for making useful protein and a peptide discover efficiently, using a nitrate reductase gene (niaD) as this amelioration promotor and a selective marker was built. Moreover, the transformation of the mold is carried out using this plasmid, and it succeeds in acquiring the transformant which has useful protein, for example, the highly productive strain of the alpha-glucosidase, or the productivity of arbitration, and came to complete this invention.

[0008] Namely, this invention (1) The enhancer DNA sequence containing array-CGGNNATTTA-, (2) The enhancer DNA sequence given in (1) given NN is GC, (3) The enhancer DNA sequence containing array-CCAATCAGCGT-, (4) The enhancer DNA sequence containing an array (1) or given in (3), (5) The enhancer DNA sequence given in (4) given NN is GC, And (6) An enhancer DNA sequence (1) or given in (3) Amelioration promotor characterized by introducing one or more into the promoterregion which functions with mold (7) The hydrolase gene of the mold origin of promoterregion, Or the amelioration promotor given in (6) which is the promoterregion of a glycolytic pathway enzyme gene, (8) Promoterregion of the alpha-glucosidase gene of the Aspergillus oryzae (Aspergillus oryzae)

origin where promoterregion is shown by array number:2, Or the amelioration promotor given in (6) which is promoterregion including the partial array, And (9) Have an amelioration promotor given in (6) and it has a suitable marker gene for selection of the transformant of host mold. The plasmid for a polypeptide manifestation in mold which has a terminator and has the DNA field which can be reproduced with Escherichia coli, (10) The nitrate reductase gene of the mold origin of a marker gene, an ornithine-carbamoyltransferase gene, The plasmid given in (9) which is a tryptophan-synthase gene or the aceto amidase gene, (11) The plasmid given in (9) a given marker gene is a nitrate reductase gene of the Aspergillus origin, (12) The terminator of the alpha-glucosidase gene of the Aspergillus oryzae origin with which a terminator is shown by array number:3, Or the plasmid given in (9) which is a terminator including the partial array, (13) To the plasmid given in (9) which has DNA which carries out the code of the polypeptide between a promotor and a terminator, and a pan (14) Manufacturing method of the polypeptide which consists of introducing a plasmid given in (13) into mold, and cultivating the obtained transformant. It provides. In addition, N shows a nucleotide during a claim and an array given in a detailed description, in an adenine and T, a thymine and C express a cytosine and G expresses [A] a guanine. Moreover, in this specification, in these arrays, unless it does not demonstrate the enhancer function of this invention even if right-hand side is the array of hard flow although shown as a three-dash terminal side, and left-hand side refuses especially a five prime end side, both enhancer sequences shall be shown with this notation. Hereafter, this invention is explained in detail.

[0009] First, the promotor of the alpha-glucosidase gene (agdA) of Aspergillus oryzae was isolated. That is, it was made from plasmid pTGF-1[publication-number No. 62868 [six to]] containing an alpha-glucosidase gene, and in order to determine all the base sequences of the alpha-glucosidase gene (agdA) containing a promotor and a terminator, the delay SHON kit for kilo sequence (TAKARA SHUZO CO., LTD. make) was used, production of a delay SHON clone was performed, and the base sequence of these clones was determined with the dideoxy chain termination method. The promoterregion (array number: 2) from all the determined base sequences, an alpha-glucosidase coding region (array number: 1), Gene-analysis software is used [a terminator field (array number: 3)] for retrieval of the enhancer nucleic-acid base sequence candidate of promoterregion after decision, respectively. The alpha-amylase gene of Aspergillus oryzae (amyB) [Biosci.Biotech.Biochem., 56, and 1849-1853 (1992)] Or it carried out by comparing with the promotor of a glucoamylase gene (glaA) [Curr.Genet., 22, and 85-91 (1992)].

[0010] In order to check the function of the enhancer candidate array found out as a result, subcloning of the promotor of an agdA gene and various delay SHON promotors (drawing 4) were built first. Next, in order to identify an enhancer sequence required for an agdA promotor's manifestation using the built various delay SHON promotors, the fusion gene which combined the beta-glucuronidase (GUS) gene (uidA) of the Escherichia coli as a reporter gene with these various delay SHON promotors was built, and the various plasmids for promotor activity measurement (drawing 2) which consist of this fusion gene, and an agdA terminator and the nitrate reductase gene (niaD) of Aspergillus oryzae as a selective marker were built. The transformation of the nitrate reductase deficit stock of Aspergillus oryzae is carried out using this plasmid by the well-known approach [Agric.Biol.Chem., 51, and 323-328 (1987)]. the Southern analysis of the obtained transformant -- carrying out -- the inside to a host's niaD locus -- homonous -- 1 -- it was copied and integrated and the transformant which can measure promotor activity correctly was chosen, without the position effect when being included in a chromosome, and being influenced of the copy number introduced. The enhancer sequence on an agdA promotor was identified by

measuring the beta-glucuronidase (GUS) activity of the these-[illegible]en transformant according to a well-known approach [Proc.Natl.Sci.USA, 83, and 8447-8451 (1986)].

[0011] That is, when an enhancer sequence existed in the XhoI-EcoRV field on an agdA promotor, the enhancer candidate array was further included all over this field and deletion of this enhancer candidate array and array of that upstream and lower stream of a river was specifically carried out from the result shown in drawing 4 , since the agdA delay SHON promotors' 6 and 7 activity decreased sharply, the array (drawing 1) which is carrying out deletion by this delay SHON promotor identified being an enhancer nucleic-acid base sequence. That is, it came to offer the enhancer nucleic-acid base sequence including the DNA sequence unit B (5'-CGGGCATTTA-3') and the base sequence unit C (5'-CCAATCAGCGT-3').

[0012] Furthermore, one DNA sequence unit E including the enhancer base sequences B and C (drawing 1) was introduced into the agdA promotor, and since it was shown that the GUS activity of an agdA promotor including a total of two DNA sequence units E increases about 3 times (drawing 5), it corroborated that this base sequence unit E had an enhancer function.

[0013] Moreover, also when which array of array E (TC) which permuted 4 and 5 base eye by array E (AA) permuted by array-AA- which exists in an amyB promotor and a glaA promotor, or the base of other arbitration, and array E (CG) was introduced from the five prime end of enhancer sequence B, it was shown that promotor activity increases more than twice (drawing 5).

[0014] From the above result, without specifying the base sequence of 4 and 5 base eye as -GC- from the five prime end of enhancer sequence B, even if it permuted by which base, it was checked that an enhancer operation is shown. That is, it was corroborated that the array A including fluctuation of a base sequence (5'-CGGNNATTTA-3') has an enhancer function. Like the above, the new enhancer nucleic-acid base sequence was offered.

[0015] Furthermore, amelioration of the promotor activity by this invention introducing the new enhancer nucleic-acid base sequence offered like **** into the promotor who functions with mold is offered.

[0016] Although it will not be restricted especially if it functions in mold as a promotor who introduces an enhancer sequence, specifically, the promotor of glycolytic pathway enzyme genes, such as hydrolase genes, such as alpha-amylase, glucoamylase, the alpha-glucosidase, a protease, lipase, a cellulase, cello BIOHAIDORAZE, and the aceto amidase, 3-phosphoglycerate kinase, a glyceraldehyde-3-phosphate dehydrogenase, and alcohol dehydrogenase, is mentioned. A suitable promotor can isolate from the gene of the alpha-amylase of an Aspergillus, glucoamylase, and the alpha-glucosidase. The promotor of the alpha-glucosidase gene of Aspergillus oryzae is mentioned more suitably. Although all the promotor's base sequences are shown by the array number 2, even if it is the partial array, as long as it has a function as a promotor in mold, they are included in this invention.

[0017] The induction to the promotor of this enhancer nucleic-acid base sequence will not be restricted especially if it is promoterregion. Moreover, as an introductory approach is the following, it can be performed. First, promoterregion to introduce is digested by two kinds of suitable restriction enzymes [A], and [B]. However, [A] uses the enzyme which produces a flush end. If there is nothing, the end of [A] will be flush-end-ized using a DNA branching kit (TAKARA SHUZO CO., LTD. make). Next, the fragment which includes an enhancer sequence using the primer which added the restriction enzyme site in order of [B] - [A], and the primer which does not add a restriction enzyme site from a five prime end is amplified by the PCR method. Thus, by inserting the amplified fragment in the promotor who

digested like ****, it is combined to a flush end site and a restriction enzyme B site, respectively. After association, although the restriction enzyme recognition site of a flush end site disappears, the site of [B] – [A] exists in another site. When introducing two or more enhancer sequences, it is finished by introducing the promotor introduced one piece by the restriction enzyme [A] and [B], and introducing an enhancer sequence one by one by the same approach after digestion and the following. Moreover, construction of a promotor with the transcriptional activity of arbitration is attained by the number of the enhancer sequence to introduce. Furthermore, as shown in drawing 6 as one concrete example, transcriptional activity not only increases the promotor of the alpha-glucosidase gene which introduced the enhancer sequence, but he did not receive a glucose repression, but it was improved by the promotor who is not influenced by the carbon source and who is constantly high-discovered. That is, it came to succeed also in amelioration of manifestation control of a promotor.

[0018] Furthermore, the new manifestation plasmid of this invention has the promotor who functions with the mold improved like ****, and a terminator, has a suitable marker gene for selection of a host's transformant, and has the DNA field which can be reproduced with Escherichia coli.

[0019] Although it will not be restricted especially if a terminator functions in mold, specifically, the terminator of the alpha-glucosidase gene of Aspergillus oryzae shown in the array number 3 or a terminator including the partial array is used more suitably.

[0020] As a desirable selective marker, the gene of nitrate reductase (niaD), ornithine carbamoyltransferase (argB), the tryptophan synthase (trpC), and the aceto amidase (amdS) is mentioned. However, the stock which does not have a functional gene about the selected selective marker must be used for host mold. A more suitable selective marker gene is a nitrate reductase gene (niaD). When using a niaD gene as a selective marker, the niaD stock of the mold which has a deficit in a gene to this marker must be used as a host. This niaD variant is acquirable by the approach [Mol.Gen.Genet., 218, and 99-104 (1989)] of anchor escapements. By using this niaD gene as a selective marker, it becomes easy to acquire the transformant integrated by the niaD locus of a host's chromosome homonous. That is, since the effect of the manifestation by the location integrated is eliminated, the manifestation of useful protein made into the purpose is [only being specified to the copy number integrated, and]. Therefore, it becomes acquirable [a transformant with the productive capacity of not only the product from Takao of useful protein but arbitration] by investigating control of the introductory number of the enhancer sequence to the promotor of the manifestation plasmid of this invention, and the copy number of the transformant chosen by the niaD gene by Southern analysis. Furthermore, for [Mol.Gen.Genet., 218, and 99-104] (1989) the reason which have a function by much mold, for example, Aspergillus nigre, Aspergillus NIDORANSU, PENISHIRYUUMU chrysoprase GENAMU, etc., an Aspergillus, for example, the niaD gene of Aspergillus oryzae, can use various mold as a host.

[0021] Construction of this plasmid can be performed by [as being the following]. The niaD gene started with the suitable restriction enzyme is inserted in an Escherichia coli vector, for example, the multi-cloning site of pUC118, from the plasmid pSTA14 containing a niaD gene [Mol.Gen.Genet., 218, and 99-104 (1989)]. Next, the terminator field to be used is started with a suitable restriction enzyme, or it amplifies by the PCR method, and inserts in the above-mentioned plasmid. The promotor who introduced the enhancer sequence built by the above-mentioned approach into the obtained plasmid is started with a suitable restriction enzyme, and it inserts in a terminator field adjacently. Thus, a manifestation

plasmid can be built. Furthermore, the plasmids pNAG136 and pNAG142 built in the procedure shown in drawing 8 as a concrete example are mentioned. These plasmids are the purposes which increase the restriction enzyme site which can be used when inserting the DNA fragment which carries out the code of the protein of the that it should be made discovered purpose between a promotor and a terminator, and have introduced eight kinds of restriction enzyme recognition sites.

[0022] This invention also indicates the approach of manufacturing mold for the useful protein or the peptide made into the purpose as a host further. This is finished by the following step. First, the suitable restriction enzyme site for the initiation codon upstream and the termination codon lower stream of a river of DNA which carry out the code of the purpose protein is introduced, respectively by the site-specific mutation method [Proc.Natl.Sci.USA, 82, and 488 (1985)] or the PCR method, and the DNA fragment which has these restriction enzyme sites in both ends is inserted in the manifestation plasmid 136, for example, pNAG, or pNAG142. Or when the terminator of the gene of purpose protein functions with mold, it is also possible to prepare a DNA fragment including a proteinic coding region and a proteinic terminator field by the same approach, and to connect it with a promotor. Thus, the transformation of the mold which has a deficit in a gene to the selective marker included in this vector is carried out using the obtained transformation vector by the well-known approach [Agric.Biol.Chem., 51, and 323-328 (1987)]. Next, this transformant is cultivated by the suitable culture medium and the culture condition after acquiring the transformant which can be grown by the selective medium to a selective marker, and the target protein is made to produce. an approach with suitable protein to have been produced -- quality -- a quantum is carried out and isolation and purification of are done if needed.

[0023]

[Example] Hereafter, although an example explains this invention concretely, the example of this invention is not restricted to these. In addition, the reaction condition of these enzymes followed the description with which the restriction enzyme used by this example and the other enzymes for genetic manipulations are attached using the product of TAKARA SHUZO or Toyobo at the time of product purchase.

[0024] example 1 the alpha-glucosidase -- a gene -- promoterregion -- a terminator -- a field -- identification -- a base sequence -- Aspergillus oryzae -- RIB -- 40 -- a share -- the alpha-glucosidase -- a gene -- containing -- 7.0 -- kbs -- PstI -- a part -- having inserted -- a plasmid -- pTGF - one -- [-- JP,6-62868,A --] -- an ingredient -- having used .

[0025] First, after acquiring the ScaI fragment of 5.0kbs containing the alpha-glucosidase from pTGF-1 and adding an EcoRI linker to this fragment, in order to determine all the base sequences of an alpha-glucosidase gene using plasmid pTGS-1 which inserted in the EcoRI part of a plasmid pUC118 in forward and reverse both directions, and was obtained, respectively, and pTGS-2, the delay SHON kit for kilo sequence (TAKARA SHUZO CO., LTD. make) was used, and creation of a delay SHON clone was performed. pTGS-1 or pTGS-2 were cut by SmaI and PstI, after digestion by Exonuclease III, flush-end-izing by mung bean nuclease, and the end restoration by Klenow fragment, ligation was carried out and, specifically, each chain length's delay SHON plasmid was created. The single stranded DNA was prepared from these delay SHON plasmids, and the base sequence was determined using automatic sequencer 370A (applied biotechnology systems company make) with the dideoxy chain termination method [Proc.Natl.Acad.Sci.USA, 74, and 5463-5467 (1977)]. Three arrays which it is checked that the coding region of an alpha-

glucosidase gene exists all over the about 3.2 kb HindIII-EcoRV under determined array, and are in agreement with [consensus sequence ["The structure and organization of nuclear genes of filamentous fungi" IRL Press of JP,6-62868,A] and the intron in mold, Oxford, and pp 93-139 (1987)] were found out. From the above result, all the base sequences containing the coding region of the alpha-glucosidase gene which consists of 985 amino acid shown in the array number 1 were determined.

[0026] Next, in order to determine the base sequence of upstream promoterregion further from the ScaI part which exists in 5' side upstream, the 1.5 kb PstI-SalI fragment of pTGF-1[JP,6-62868,A] was inserted in PstI of a plasmid pUC118 or the multi-cloning part of pUC119, and a SalI part. The single stranded DNA was prepared from the plasmid, and the base sequence was determined using the automatic sequencer with the dideoxy chain termination method like the time of a delay SHON plasmid. The base sequence of the promoterregion of 720bp(s) shown in the array number 2 from the above result was determined.

[0027] Moreover, from the termination codon (TGA) of an alpha-glucosidase gene, die length sufficient in the 5.0kb ScaI fragment determined by the delay SHON plasmid is contained, and the base sequence of the terminator field of 3' lower stream of a river shows the base sequence of the terminator field of 840bp(s) from termination to the array number 3.

[0028] Example 2 In order to identify the strange enhancer sequence which exists all over the promoterregion of a new enhancer candidate's retrieval alpha-glucosidase gene (agdA), first, DNASIS gene-analysis software (Hitachi Software Engineering Co., Ltd. make) was used, and homology retrieval of a promotor was performed. the promotor [Biosci.Biotech.Biochem., 56, 1849-1853 (1992)] of the alpha-amylase gene (amyB) of Aspergillus oryzae by whom a high manifestation and high-secreting are generally known -- or The place compared with the promotor [Curr.Genet., 22, and 85-91 (1992)] of a glucoamylase gene (glaA), Although the homology as the whole promoterregion showed the promotor of amyB, the promotor of 51% and glaA, and 52% of homology, the high field of especially a homology was not found. then -- next, when the promotor of agdA was divided per 20-50bp and more detailed homology retrieval was carried out, the array shown in drawing 1 (5'-CGGGCTAAAT-3') and the array (5'-CGGAAATTTA-3') of a high homology were discovered by the amyB promotor and the glaA promotor. Moreover, it was checked that two well-known fields RegionI and II [Curr.Genet., 22, and 85-91 (1992)] saved in common by the amyB promotor and the glaA promotor in this process exist in agdA promoterregion.

[0029] Example 3 In order to identify the existence or the other enhancer part of the enhancer operation of an array (5'-CGGGCTAAAT-3') found in construction homology retrieval of an alpha-glucosidase gene promotor's subcloning and various delay SHON promotors, the PCR method was used and an agdA promotor's subcloning was performed.

[0030] As an upper primer which added the PstI site in order to amplify 702bp(s) of the agdA promotor's existence locations 17-718 shown in the array number 2, he is 5SP. : As a down-stream primer which added the 5'-GGCTGCAGTCATGGCACCACTAGAGATG-3' SalI site, it is 3ASP. : 5'-CCGTCGACCGTGGTCCGCCAAGTTGATT-3' was compounded using DNA synthesis machine model 391 PCR-MATE (applied biotechnology systems company make). The plasmid pAGP1 (drawing 1) which acquires an agdA promotor's PstI-SalI fragment, inserts this fragment in the multi-cloning site of pUC118, and contains an agdA promotor by thermal SAIKURA (PerkinElmer Japan Co., Ltd. make) was acquired using plasmid pTGF-1 as above-mentioned two primers and templates.

[0031] Next, the various delay SHON promotors of agdA as shown in drawing 4 using this pAGP1 were built. After digesting pAGP1 by PstI and EcoT14I, specifically, the about 3.8 kb PstI-EcoT14I fragments which covered over agarose electrophoresis 0.8% and were separated were collected using the GeneClean kit (product made from 101 companies of biotechnology). The plasmid pAGP2 including the agdA delay SHON promotor 2 (P-agdA2) was acquired by carrying out self ligation of this fragment after flush-end-izing using a DNA branching kit (TAKARA SHUZO CO., LTD. make). Hereafter, after carrying out duplex digestion of pAGP1 by PstI, XhoI, EcoRV, or FbaI, the agdA delay SHON promotors 3 and 4, the plasmid 3 and pAGP 4 containing 5, or 5 was acquired by the same approach, respectively.

[0032] The agdA delay SHON promotor 6 (P-agdA6) who made the amyB promotor found as a result of homology retrieval and the glaA promotor do deletion only of homology's high base sequence (5'-CGGGCTAAAT-3') acquired using the combination PCR method ["PCRTechnology", Stocton Press, New York, and pp.61-70 (1989)]. the internal upstream primer which specifically carried out deletion of the high homology array first -- carrying out -- 5ISP: 5 -- '-GCGTCGTGTCGGGGGATGGACCAATCAGC-3' -- the same -- as an internal down-stream primer -- 3IASP: 5'-CATCCCCCGACACGACGCTTGAGCCCTGA-3' was compounded.

[0033] Next, the PCR product 1 was amplified using upper primer 5SP and internal down-stream primer 3IASP which were compounded at the time of an agdA promotor's subcloning, and, similarly the PCR product 2 was amplified using internal upstream primer 5ISP and down-stream primer 3ASP. The acquired PCR products 1 and 2 were mixed, it considered as the template, and the 3rd PCR reaction was performed in the following reaction solution using Gene AmpTM kit (PerkinElmer Japan Co., Ltd. make) using upper primer 5SP and down-stream primer 3ASP.

H2O A 70.5microl10x reaction solution 10microldNTP mix (1 25mM) 16microl upstream primer 5SP (0.1mM) 1microl down-stream primer 3ASP (0.1mM) The 1microlPCR product 1 The 1microl(5-100ng) PCR product 2 1microl(5-100ng) AmplitaqTMDNA polymerase 0.5microl reaction condition is ->(94-degree-C, 0.5 minutes) (55-degree-C, 1 minute) -> (72 degrees C, 2 minutes), and is 25 cycle ******. After collecting the reaction solutions after PCR magnification by ethanol precipitation after phenol chloroform processing and processing them by PstI and SalI, agarose electrophoresis was presented with them 0.8%, and they isolated and refined the agdA delay SHON promotor 6 (P-agdA6). The plasmid pAGP6 which inserts in pUC118 which processed this delay SHON promotor by PstI and SalI, and contains P-agdA6 was acquired.

[0034] The agdA delay SHON promotor 7 (P-agdA7) of homology's high base sequence as shown in drawing 1 who did deletion only of the upstream region 1 to the downstream region 3 immediately, or 8 (P-agdA8) was acquired using the same combination PCR method, respectively, it inserted in PstI and pUC118 which carried out SalI processing, respectively, and pAGP7 or pAGP8 was acquired. Moreover, the delay SHON promotor who got by the combination PCR method checked that the target array was carrying out deletion by investigating a base sequence with a dideoxy chain termination method.

[0035] Example 4 In order to identify an enhancer sequence required for an agdA promotor's manifestation using the various agdA delay SHON promotors by whom construction construction of the plasmid for promotor activity measurement was done, the plasmid pNAGT4 (drawing 2) for promotor activity measurement which consists of an agdA terminator, a nitrate reductase gene (niaD) of Aspergillus oryzae as a selective marker, and a beta-glucuronidase (GUS) gene (uidA) of the Escherichia coli (E.coli) as a reporter gene

was built.
[0036] First, agarose electrophoresis was presented with the plasmid pSTA14 [Mol.Gen.Genet., 218, and 99-104 (1989)] containing a nitrate reductase gene (niaD) 0.8% after digestion by XhoI and HindIII, and the XhoI-HindIII fragment of 4.4kbs containing niaD was isolated and refined. After flush-end-izing this fragment using a DNA branching kit (TAKARA SHUZO CO., LTD. make), it inserted in pUC118 similarly flush-end--ization-processed after EcoRI processing, and pNR10 was obtained.
[0037] Next, in order to insert a beta-glucuronidase (GUS) gene (uidA) in pNR10 It digests to the SmaI site of 5' upstream of the GUS gene of pBI221 [Proc.Natl.Acad.Sci.USA, 83, and 8447-8451 (1986)]. After attaching and carrying out self ligation of the SalI linker to this next, to the SacI site of 3' lower stream of a river, after digestion, it flush-end-ized, the XbaI linker was attached, self ligation was carried out similarly, and pBI221-SX was obtained. After using agarose electrophoresis and isolating and refining similarly the SalI-XbaI fragment of about 1.9 kbs which contain a GUS gene from this pBI221-SX, it was inserted in SalI of the multi-cloning site of pNR10, and a XbaI site, and the plasmid pNRG1 was obtained.
[0038] In order to insert the terminator (T-agdA) of an alpha-glucosidase gene (agdA) in this pNRG1, the terminator was first acquired by the PCR method. As an upper primer which added the XbaI site in order to amplify 830bp(s) of the agdA terminator's shown in array number 3 existence locations 8-837, it is 5TSP. : As a down-stream primer which added the 5'-CCTCTAGAAGCGTAACAGGATAGCCTAG-3' SmaI site, it is 3TASP. : 5'-GGCCCGGGAGTAACCCATTCCCGGTTCT-3' was compounded using DNA synthesis machine model 391 PCR-MATE (applied biotechnology systems company make). The XbaI-SmaI fragment of an agdA terminator was acquired by thermal SAIKURA (PerkinElmer Japan Co., Ltd. make), using plasmid pTGF-1 as the two above-mentioned primers and templates. This fragment was inserted in XbaI and pNRG1 which carried out SmaI digestion, and the plasmid pNAGT4 for promotor activity measurement was obtained.
[0039] Example 5 The transformant of Aspergillus oryzae was acquired using the plasmid which inserted various agdA delay SHON promotors in pNAGT4 obtained as acquisition above-mentioned [of a transformant], and was obtained.
[0040] First, pAGP 2, 3, 4, and 5 isolated and refined the SalI-NaeI fragment which contains a delay SHON promotor after digestion using agarose electrophoresis by SalI and NaeI among plasmids including the various above-mentioned agdA delay SHON promotors. Moreover, the SalI-PstI fragment including a delay SHON promotor was similarly isolated and refined from pAGP 1, 6, 7, and 8 processed by SalI and PstI.
[0041] Next, eight kinds of plasmids of pNAGG 1-1 to pNAGG 1-8 were obtained by introducing into pNAGT4 each delay SHON promotor fragment corresponding to the restriction enzyme which used pNAGT4 after isolating and refining SalI, NaeI digestion or SalI, and pNAGT4 that carried out PstI digestion and was similarly processed with each restriction enzyme, respectively. The example is shown in drawing 2 .
[0042] Next, the transformation of Aspergillus oryzae by the plasmid of pNAGG1 series obtained above was performed. After carrying out shaking culture of 30 degrees C of 14 shares of nitrate reductase deficit stocks of Aspergillus oryzae (niaD-</SUP>), for example, the niaD, [Mol.Gen.Genet., 218, and 99-104 (1989)] for three days by the dextrin peptone medium (2% a dextrin and 1% the poly peptone, 0.5% KH2PO4, 0.05% MgSO4.7H2O), sterilized water washed the obtained fungus body. This fungus body was suspended in the cell wall solution [10mM phosphate buffer solution, pH 6.0, 0.8M NaCl, and 20mg/ml YATARAZE (TAKARA SHUZO CO., LTD. make)], and 30 degrees C was protoplast-ized by shaking

gently for 2 to 3 hours. The fungus body which remains by filteri[redacted]he obtained protoplast with a glass filter was removed.
[0043] Next, preparation and the transformation of a competent cel are performed using this protoplast by Gomi's and others approach [Agric.Biol.Chem. and 51,323-328 (1987)]. the culture medium which contains a nitric acid as a single nitrogen source, for example, Czapek-Dox agar, (it KCl(s) 0.2% NaNO3 and 0.1% K2HPO4 -- 0.05%) 0.05% MgSO4.7H2O and 2% A total of a glucose and every 12 shares each per each plasmid of 96 shares of transformants which can be grown by pH 5.5] was acquired.
[0044] example 6 the Southern analysis of various delay SHON promotors' beta-glucuronidase activity profit **** various transformants -- carrying out -- from the inside -- homonous -- 1 -- the stock copied and integrated was chosen and beta-glucuronidase (GUS) activity was measured.
[0045] First, Chromosome DNA was prepared in order to perform Southern analysis. Conidium 1 platinum loop of a transformant was inoculated into 100ml (2% a dextrin and 1% the poly peptone, 0.5% KH2PO4, 0.1% NaNO3, 0.05% MgSO4.7H2O) of dextrin peptone media, for 30 degrees C and three days, after shaking culture, the obtained fungus bodies were collected with 3G1 glass filter, and sterilized water washed. After the filter paper removed the moisture of this fungus body, it mashed in liquid nitrogen using the mortar beforehand cooled at -80 degrees C. After suspending this fungus body debris in TE solution (10 mM Tris-HCL, 1mM EDTA, pH 8.0), 37 degrees C (0.5% SDS, 50mM EDTA) of bacteriolysis solutions were left for 30 minutes after churning equivalence, in addition gently. Supernatant liquid was acquired for the obtained lysate after cooling centrifugal separation for 3000rpm and 20 minutes. After processing the supernatant liquid twice with equivalent phenol chloroform mixed liquor and removing the protein with which are contaminated, the cold ethanol of ** was added 2.5 times and DNA was settled. This precipitate was gently dissolved in TE solution containing 0.1mg [/ml] RNase, and 30 degrees C reacted for 30 minutes. After carrying out phenol chloroform processing of this solution, the cold ethanol of ** was added 2.5 times and the produced chromosome DNA was rolled round with Pasteur pipette. It dissolved in TE solution after drying rolled-round DNA, and the chromosome DNA solution was prepared. After after digestion and agarose electrophoresis having separated by SalI and carrying out the blot of the obtained chromosome DNA to the nylon membrane (Amersham make) N, for example, high bond, Southern analysis was performed by using a plasmid pUC118 as a probe. At this time, labeling of a probe and detection of a signal were performed using ECL random prime DNA labeling and a detection system (Amersham make). Consequently, two kinds of patterns in case it hybridizes also from 11.0kbs equivalent to the size of a plasmid to 10.6kbs in addition to detection of the single band of 9.3kbs to 8.9kbs depending on each delay SHON promotor's size contained in the plasmid used for the transformation or this single band and a total of two bands are detected existed. As this result is shown in drawing 3 , in the case of one band, it means being integrated by 2 or more ******s and a host's niaD locus homonous in the case of one copy and two bands.
[0046] When the niaD gene is included as a selective marker gene and carries out a transformation by this selective marker independent, the plasmid used for the transformation [Mol.Gen.Genet., 218, and 99-104] which are integrated homonous by high frequency (1989), although things are clear Also when a transformation was carried out to this using the plasmid containing another gene to newly introduce, being integrated homonous by the niaD locus became clear, without being influenced of the gene introduced. Moreover, recombination by double crossover was produced in the niaD locus very rarely,

and the pattern with which only a niaD gene is integrated also ex[illegible]d.
[0047] From the result of Southern analysis, it made into arbitration at a time two shares of transformants which can measure promotor activity correctly a total of 16 hill selections per [which was used] plasmid, without the position effect of the transformant suitable for measurement of promotor activity, at i.e., the only one copy being integrated homonous and included in a chromosome time, and being influenced of the copy number introduced.
[0048] Next, 16 shares of 5x105 selected conidia were inoculated into 15ml (1% the poly peptone, 0.1% K2HPO4, 0.05% MgSO4.7H2O, 0.05% KCL, 0.01% FeSO4, and 2% a glucose, pH 5.5) of TSUAPEKKU DOKKUSU Pori peptone media, and the obtained fungus bodies were collected with 3G1 glass filter after shaking culture for 30 degrees C and three days. After the filter paper removed the moisture of this fungus body, about 0.2g fungus body was mashed in liquid nitrogen using the mortar cooled at -80 degrees C. The supernatant liquid which carried out centrifugal separation and removed the fungus body residue was used [after extracting an enzyme] as the enzyme solution for 15 minutes 15000 rpm by agitating violently for about 1 minute after suspending this fungus body debris in a 0.8ml extract solution (50mM phosphate buffer solution, pH 7.0, 10mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10mM beta-mercaptoethanol). The beta-glucuronidase (GUS) activity in the obtained enzyme liquid It applies to a well-known approach [Proc.Natl.Acad.Sci.USA, 83, and 8447-8451 (1986)] correspondingly. a 1ml reaction solution (50mM phosphate buffer solution, pH 7.0, 0.1% Triton X-100, and 10mM beta-mercaptoethanol --) Enzyme liquid 5microl to 50microl is made to react at 37 degrees C in 1mM p-nitrophenyl glucuronide, and it is 415nm. It carried out by measuring an absorbance. In addition, the relative activity which makes 100% GUS activity produced by the original alpha-glucosidase promotor (P-agdA1) who has not done delay SHON showed the activity at this time. The result is shown in drawing 4 .
[0049] When deletion of the agdA promotor was carried out from 5' upstream region, the big activity fall at two places of the field (XhoI-EcoRV) between the field between P-agdA1 and P-agdA2 (PstI-EcoT14I), P-agdA3, and P-agdA4 was seen. The inside of it, and all over the PstI-EcoT14 I region, the well-known conserved sequence RegionI [Curr.Genet., 22, and 85-91 (1992)] existed. Moreover, all over the XhoI-EcoRV field, the base sequence B (5'-CGGGCATTTA-3') found out as a result of homology retrieval was included. Furthermore, since the activity of the delay SHON promotor (P-agdA6) who did deletion of the above-mentioned array B was also decreasing sharply, it was checked that the base sequence B which exists in an agdA promotor is a new enhancer. Moreover, the relative activity of P-agdA7 was shown only 19% among promotor P-agdA7 which carried out deletion only of the base sequence before and behind enhancer sequence B, respectively, and P-agdA8, but it was checked that the array C (5'-CCAATCAGCGT-3') which carried out deletion here is an enhancer sequence.
[0050] example 7 the decision of the versatility of a new enhancer sequence -- the base sequence B (5'-CGGGCATTTA-3') by which it was checked that it is a new enhancer sequence, and a highly preservable array In order to check existing also in an alpha-amylase promotor and a glucoamylase promotor as a result of the homology retrieval performed in the example 2 and to check whether these arrays also have an enhancer function The promotor who inserted in the agdA promotor the array which permuted a different base part from Array B by the base of arbitration was created, and the promotor activity was measured.
[0051] First, after carrying out restriction enzyme processing of an agdA promotor's EcoRV and ClaI site using the plasmid pAGP1 including the agdA promotor (0.7 kb PstI-SalI

fragment) who acquired in the example 3, it flush-end-ized using DNA branching kit (TAKARA SHUZO CO., LTD. make), and the plasmid pAGP131 including the agdA delay SHON promotor 131 (P-agdA131) who removed the EcoRV-ClaI fragment which does not include an enhancer sequence was acquired by carrying out self ligation (drawing 5). Next, in order to insert in the change of an EcoRV-ClaI fragment which removed DNA sequence E (a concrete array is shown in drawing 4) including the enhancer sequences B and C shown in drawing 5 , this array E was acquired by the PCR method. Specifically, it is 5SPE as an upper primer. : As a down-stream primer which added the 5'-TCAAGCGTCGTGTCGGGCATT-3' ClaI site, it is 3ASPE. : 5'-CCATCGATGATATCCCTACGCTGATTGG-3' was compounded using DNA synthesis machine model 391 PCR-MATE (applied biotechnology systems company make). It amplified by thermal SAIKURA (PerkinElmer Japan Co., Ltd. make), using plasmid pTGF-1 as above-mentioned two primers and templates. In order to insert this amplified array E in an agdA promotor, the agdA promotor was digested by EcoRV and ClaI like the time of P-agdA131, and the EcoRV-ClaI fragment was removed using agarose electrophoresis. Plasmid pAGP132-GC containing promotor 131GC (P-agdA132GC) which inserted Array E was obtained by carrying out ligation of the array E to this.

[0052] Next, in order that 4 base eye and 5 base eye might insert in an agdA promotor array E (AA) including the array permuted by -AA- (5'-CGGAAATTTA-3'), i.e., the array common to an alpha-amylase (amyB) promotor and a glucoamylase (glaA) promotor, like P-agdA131GC from the five prime end of enhancer sequence B, PCR magnification of array E (AA) was performed. As an upper primer, it is 5 SPE-AA. : 5'-TCAAGCGTCGTGTCGGAAATT-3' was compounded and the down-stream primer was amplified by thermal SAIKURA like former using primer 3ASPE used by array E magnification. This amplified array E (AA) was inserted in the agdA promotor by the same approach as the time of P-agdA132GC, and plasmid pAGP132-AA containing promotor 132AA (P-agdA132AA) was acquired. Besides it, moreover, 4 base eye and 5 base eye from the five prime end of enhancer sequence B - Be by the same approach as **** about array E (TC) including the array permuted by TC- or -CG-, or array E (CG). After amplifying using PCR, It inserted in the agdA promotor and promotor 132TC (P-agdA132TC), plasmid pAGP132-TC containing promotor 132CG (P-agdA132CG), or pAGP132-CG was acquired.

[0053] Agarose electrophoresis isolated and refined the PstI-SalI fragment which contains various alteration agdA promotors for five kinds of plasmids obtained above after processing by PstI and SalI. It inserted in PstI of the plasmid pNAGT4 (drawing 2) for promotor activity measurement which built this fragment in the example 4, and a SalI site, and pNAGG 1-131, -132GC, -132AA, -132TC, and -132CG were obtained. A total of every six shares each of 30 shares of transformants of Aspergillus oryzae was acquired about each plasmid by the approach which used these plasmids and was shown in the example 5 below. the Southern analysis of these transformants -- the approach of an example 6 -- carrying out -- a nitrate reductase gene (niaD) seat -- homonous -- 1 -- it was copied and integrated and chose two shares of transformants which are not influenced of the position effect included in a chromosome, and the copy number introduced as arbitration at a time. According to the approach of an example 6, after cultivating by the TSUAPEKKU DOKKUSU Pori peptone medium, an alteration promotor's activity was measured by performing fungus body crushing according the selected transformant to liquid nitrogen, extract of the enzyme from a crushing fungus body, and beta-glucuronidase (GUS) activity measurement of extract enzyme liquid.

[0054] Consequently, the GUS activity of alteration promotor 132GC (P-agdA132GC) which

introduced DNA sequence E including enhancer sequences B and into the agdA promotor showed the 2.7 times as many rise as this compared with P-agdA131 of control, and it was checked that the array E including enhancer sequences B and C has the capacity which reinforces promotor activity. Moreover, also when which array of array E (TC) which permuted 4 and 5 base eye by array E (AA) permuted by array-AA- which exists in an amyB promotor and a glaA promotor, or the base of other arbitration, and array E (CG) was introduced from the five prime end of enhancer sequence B, it was shown that promotor activity increases more than twice. From the above result, even if it permuted by which base, without specifying the base sequence of 4 and 5 base eye as -GC- from the five prime end of enhancer sequence B, it was checked that an enhancer operation is shown. That is, it was shown that the array A including fluctuation of a base sequence (5'-CGGNNATTTA-3') has an enhancer function.

[0055] Example 8 Promotor activity was improved by introducing two or more enhancer sequence A (5'-CGGNNATTTA-3') including fluctuation of amelioration 2 base of the promotor by installation of an enhancer sequence, and DNA sequence E containing enhancer sequence C into an agdA promotor.

[0056] First, promotor P-agdA132GC built in the example 7 is the promotor into whom one array E was introduced, the five prime end of the array E introduced at this time is combined by the flush end, and the recognition site after [EcoRV] association disappears. On the other hand, since the ClaI site is added at the time of PCR in addition to the EcoRV site included in Array E from the first, as a three-dash terminal is shown in drawing 5 or 6 after association, two restriction enzyme sites exist. Sequential installation of two or more arrays E was carried out at the tandem using this EcoRV and a ClaI site. Plasmid pAGP132-GC containing P-agdA132GC was specifically digested by EcoRV and ClaI, the array E which has EcoRV and a ClaI site at the three-dash terminal which carried out PCR magnification in the example 7 was introduced into this, and pAGP133 was built. This pAGP133 was digested by EcoRV and ClaI, like the following, the sequential array E is introduced into the promotor, a maximum of 11 pieces were introduced, and the plasmid even containing a total of 12 pieces and promotor P-agdA142 to which Array E exists in a tandem was built. Four arrays E six plasmids 134, 136, and pAGP 142 including the promotor whose 12 pieces exist out of these plasmids PstI, Agarose electrophoresis isolates and refines these amelioration promotors after digestion by SalI. PstI, PstI of the plasmid pNAGT4 (drawing 2) for promotor activity measurement which built the amelioration promotor who has a SalI site in both ends in the example 4, and a SalI site -- inserting -- pNAGG 1-134 and - 136 and 142 were acquired.

[0057] Next, the plasmid which connected the GUS gene as a reporter gene with this was built like an agdA promotor's case, using the promotor of the glucoamylase gene (agdA) of Aspergillus oryzae as improved contrast of an agdA promotor. The primer shown below concrete first from a well-known glaA promotor base sequence [Curr.Genet., 22, and 85-91 (1992)] was compounded, and subcloning of the glaA promotor (P-glaA) was carried out. As an upper primer which added the PstI site, it is 5SGP. : As a down-stream primer which added the 5'-GGCTGCAGAGCCTACGCTAAAGCAAAGT-3' SalI site 5ASGP(s): 5 -- '-CCGTCGACTGCTTCGACTTCGTTTGCTG-3' -- the plasmid which contains a glaA promotor (P-glaA) using these primers -- For example, PCR magnification is performed by making pRGA-1[Gene, 108, and 145-150(1991)] into a template. A galA promotor's (P-glaA) 0.7 kb PstI-SalI fragment was acquired, it inserted in PstI of the plasmid pNAGT4 for promotor activity measurement built in the example 4, and a SalI site, and pNGAG1 was built. next, the approach shown in the example 5 using these plasmids -- a transformant --

every six shares each per each plasmid of a total of 24 shares -- acquiring -- the Southern analysis of these transformants -- the approach of an example 6 -- carrying out -- a niaD locus -- homonous -- 1 -- it chose as arbitration at a time two shares of transformants copied and integrated. An amelioration promotor's activity was measured by performing fungus body culture and the extract of an enzyme according to the approach of an example 6 from the selected transformant, and performing beta-glucuronidase (GUS) activity measurement of extract enzyme liquid.

[0058] Consequently, GUS activity increased and enhancement of one 3.5 times the promotor activity of this was checked in P-agdA142 compared with P-agdA131 in which one array E exists as were shown in drawing 6 and Array E was introduced into the promotor at the tandem. Furthermore, it was checked that the approach of showing the promotor activity of 3 times or more even if originally compared with a glaA promotor useful as a high manifestation promotor, and introducing an enhancer sequence into a promotor is very effective.

[0059] Example 9 In order to examine the effectiveness over the carbon source of promotor P-agdA142 formed into the high manifestation by introducing two or more effectiveness enhancer sequence E to an amelioration promotor's carbon source into a tandem, the GUS activity of the fungus body grown by the culture medium including various carbon sources was measured.

[0060] The fusion gene which connected the GUS gene with the original agdA promotor before the amelioration used in the example 6 (P-agdA1) One copy, The amelioration promotor who used it in the transformant and example 8 which are integrated (P-agdA142) The fusion gene of a GUS gene with a glaA promotor (P-gla) Or one copy, the transformant integrated, two shares of each, and a total of six shares of 1x106 conidia -- a TSUAPEKKU DOKKUSU Pori peptone medium (1% poly peptone --) 0.1% K2HPO4, 0.05% MgSO4.7H2O, 0.05% KCl, 0.01% FeSO4, and 2% A glucose, pH 5.5 Or 15ml of culture media permuted by various kinds of carbon sources which show the glucose of this culture medium in Table 1 was inoculated, for 30 degrees C and three days, according to the approach of an example 6, fungus body crushing and an enzyme extract were performed after shaking culture, and the GUS activity of extract enzyme liquid was measured. The result is shown in Table 1.

[0061]

[Table 1]

炭素源	GUS活性 (U/mg-protein)		
	P-agdA1	P-agdA142	P-glaA
グルコース	57.3	614	236
マルトース	130	864	700
マルトトリオース	109	706	596
イソマルトース	146	799	645
可溶性澱粉	144	820	670

[0062] The amelioration promotor (P-agdA142) showed five to 6 times, and high activity by 10 or more times and other carbon sources by the glucose compared with the original promotor (P-agdA1). Furthermore, P-agdA142 showed high activity also in which carbon source offered as a sample even if compared with the glaA promotor (P-glaA). Moreover, although the activity at the time of a carbon source glucose had the description which falls

extremely since a glaA promotor or an original agdA promotor was influenced of a glucose repression, in the amelioration promotor, about 80% of relative activity of other carbon sources was held also in the glucose. Promotor P-agdA142 improved when this result introduced two or more enhancer sequences was not influenced by the carbon source, but it meant that it was constantly high-discovered, and that usefulness was very high and a promotor's manifestation control function also showed that it was improvable.

[0063] Example 10 Although the remarkable increment in GUS activity was checked by amelioration promotor P-agdA142 built in an amelioration promotor's Northern analysis example 8, it checked whether it was a sake, the increment in a promotor's transcriptional activity, i.e., the increment in mRNA of a GUS gene (uidA), of this increment in activity.

[0064] the fusion gene of the promotor (P-agdA134) who exists four promotors (P-agdA131) in whom only one enhancer sequence E built in the example 8 exists or the amelioration promotor (P-agdA142) whose 12 pieces exist, and a GUS gene -- 1 -- 1x106 conidia of the transformant copied and integrated were inoculated into 100ml of TSUAPEKKU DOKKUSU Pori peptone media, for 30 degrees C and three days, the harvest was carried out with the glass filter after shaking culture, and sterilized water washed. After the carbon source moved about 2.0g of washing fungus bodies to a glucose or 100ml of TSUAPEKKU DOKKUSU Pori peptone media of a maltose, 30 degrees C of fungus bodies which carried out shaking culture for 12 hours were further collected with the glass filter, and sterilized water washed. The moisture of this washing fungus body is mashed in liquid nitrogen using the mortar which cooled about 2.0g fungus body at -80 degrees C after removal through the filter paper. this fungus body debris -- a 6ml guanidine isothiocyanate solution (5M guanidine isothiocyanate --) 10mM EDTA, 50 mM Tris-HCl, and pH 7.5 and 0.6ml beta-mercaptoethanol were added, and RNA was prepared after adjusting a suspension solution according to the well-known approach [DNA, 2, and 329-335 (1983)]. After carrying out the transformer blot of the 20microg of this RNA to the nylon membrane (Amersham make) N, for example, high bond, after separation by formaldehyde agarose gel electrophoresis, RNA was fixed on the membrane by carrying out UV irradiation using Tran Swi Rumi Noether. Next, after making 42 degrees C of the GUS genes (uidA) and membranes of the 1.9 kb XbaI-SalI fragment prepared from plasmid pBI221-SX (drawing 2) hybridize for 17 hours in a hybridization solution (5xSSPE, 5 xDenhardt's solutions, and 50% a formamide, 0.5% SDS, and 100microg/ml thermal denaturation salmon sperm DNA), the membrane was washed by the suitable washing solution, for example, 6xSSC, or 0.2xSSC, and 0.1% SDS. This membrane and the hybridized signal were detected on the X-ray film.

[0065] consequently, it is shown in drawing 7 -- as -- a carbon source glucose and a maltose -- mRNA of a lot of [the promotors (P-agdA142) to whom 12 enhancer sequences exist from one promotor (P-agdA131) in the case of which] GUS genes (uidA) was detected. It meant that a promotor's transcriptional activity increased this by introducing two or more enhancer sequences into an agdA promotor, and it was checked that the increment in GUS activity is the result of having improved the promotor function of P-agdA142.

[0066] Example 11 It had the construction nitrate reductase gene (niaD) of the quantity expression vectors pNAG136 and pNAG142 in the selective marker, and built in the high expression vector pNAG136 with the versatility which introduced the multi-cloning site (MCS) between high manifestation promotor P-agdA136 or P-agdA142, and an agdA terminator, or the procedure which shows pNAG142 in drawing 8 .

[0067] First, the down-stream primer, 3TASP-2 which added upper primer 5TSP which added the XbaI site compounded in the example 4 in order to amplify 578bp(s) of the agdA

terminator's shown in array number 3 existence locations 8-585 and a SmaI site : 5'-GGGAGGTGTACGCTTGGTAAAGT-3' was compounded using DNA synthesis machine model 391 PCR-MATE (applied biotechnology systems company make). PCR magnification was performed by having made plasmid pTGF-1[JP,6-62868,A] into the template using these primers, and the 0.6 kb XbaI-SmaI fragment of an agdA terminator was acquired. It inserted in XbaI of the multi-cloning site (MCS) of the plasmid pNR10 containing the niaD gene which built this fragment in the example 4, and a SmaI site, and the plasmid pNRT10 was built. The PstI-SalI fragment of six pieces, high manifestation promotor P-agdA136 which it has 12 pieces, or P-agdA142 was inserted in PstI of this plasmid, and a SalI site for the enhancer sequence built in the example 8, respectively, and pNAG136xs or pNAG142xs was acquired.

[0068] Next, in order to introduce a multi-cloning site between an agdA terminator (T-agdA) and a high manifestation agdA promotor (P-agdA136 or P-agdA142), the restriction enzyme usable as a multi-cloning site was searched first. That is, as a result of searching the niaD gene which is the component of a high expression vector, an agdA terminator (T-agdA), a high manifestation promotor (P-agdA136 or P-agdA142), and the restriction enzyme that exists in neither of pUC118, it was newly in addition to XbaI and SalI which can be used by pNAG136xs or pNAG142xs checked that NdeI, NotI, PmaCI, and SpeI can be used. Moreover, if HindIII and SphI which exist in a high manifestation agdA promotor's five prime end are removed, since these restriction enzymes can also be used, after digesting pNAG136xs or pNAG142xs by HindIII and SphI and flush-end-izing using a DNA branching kit (TAKARA SHUZO CO., LTD. make), HindIII and a SphI site were removed by carrying out self ligation. Next, as a result of above-mentioned retrieval, the sense chain and the antisense strand were compounded using the DNA synthesis machine, respectively, annealing was carried out by ice-cooling the multi-cloning site (drawing 8) which consists of eight kinds of usable restriction enzymes (both ends are XbaI and SalI) after [after mixing these] -> (for 94-degree-C and 1 minute) (for 55-degree-C and 10 minutes) incubation, and the multi-cloning site of 2 chains was acquired. The compound single stranded DNA is shown below.

[0069] Sense chain: 5'-CTAGAGCATGCCATATGACTAGTCACGTGGCGGCCGCAAGCTTG-3' antisense strand: 5'-TCGACAAGCTTGCGGCCGCCACGTGACTAGTCATATGGCATGCT-3' [0070] After phosphorizing the five prime end of the multi-cloning site obtained here using T-four polynucleotide kinase (TAKARA SHUZO CO., LTD. make), pNAG136 and pNAG142 were built by inserting in XbaI of pNAG136xs which removed HindIII and SphI, and pNAG142xs, and a SalI site.

[0071] Example 12 In order to carry out the breeding of the alpha-glucosidase (AGL) highly productive strain by P-agdA136 improved by the high manifestation promotor by introducing into an agdA promotor two or more enhancer sequence E built in the acquisition example 8 of the alpha-glucosidase highly productive strain of Aspergillus oryzae, or P-agdA142, the plasmid pNAGL136 for transformations or pNAGL142 was built first. The construction procedure is shown in drawing 9 .

[0072] Agarose electrophoresis isolated and refined after digestion the plasmid pAGP136 containing amelioration promotor P-agdA136 built in the example 8, or P-agdA142, or pAGP142 by CpoI and BamHI. Next, plasmid pTGF-1[JP,6-62868,A] containing an alpha-glucosidase gene (agdA) was processed by CpoI and BamHI, and the 3.8 kb CpoI-BamHI fragment including the coding region and terminator field of agdA was isolated and refined similarly. This fragment was inserted in the above-mentioned CpoI and the plasmid which

carried out BamHI processing, and pAGL136 or pAGL142 was b[redacted]The transformation plasmid pNAGL136 or pNAGL142 was built by isolating and refining the PstI-SmaI fragment of an agdA gene with which these plasmids were connected with the amelioration agdA promotor of 4.4kb or 4.7kb after digestion by PstI and SmaI, and inserting in PstI of the plasmid pNR10 containing the nitrate reductase gene (niaD) as a selective marker which built this fragment in the example 4, and a SmaI site.

[0073] Next, the transformation of the nitrate reductase deficit stock (nia14) of Aspergillus oryzae was performed by the approach shown in the example 5 using these plasmids, and it carried out by the approach of describing screening of a highly productive strain below, after acquiring about 300 shares of transformants. the TSUAPEKKU DOKKUSU agar medium (it KCl(s) 0.2% NaNO3 and 0.1% K2HPO4 -- 0.05%) which contains a 0.05%4-nitrophenyl-alpha-D-glucoside (4NPG) for conidium 1 platinum loop of a transformant 0.05% MgSO4.7H2O and 2% A glucose and 1.5% Agar, Screening was performed for the strength of the time amount which begins to change to yellow for 4-nitrophenol which pH 5.5 were inoculated, and 4NPG(s) which are substrates were decomposed by the operation of the alpha-glucosidase by which the surrounding color of the grown fungus body colony was secreted, and was produced, and a color against the index. By this screening, about ten shares with strong activity were chosen, next Southern analysis of these transformants was performed, and most stocks of a copy number were screened. Moreover, Southern analysis was performed to coincidence also about several shares of arbitration with weak activity. In addition, preparation of Chromosome DNA was performed according to the approach of an example 6. After agarose electrophoresis's having separated after digestion the chromosome DNA obtained here by XhoI and SalI and carrying out a blot to a nylon membrane (Amersham make) N, for example, high bond, Southern analysis was performed by using the agdA gene of a 3.8 kb CpoI-BamHI fragment as a probe. At this time, labeling of a probe and detection of a signal were performed using ECL random prime DNA labeling and a detection kit (Amersham make).

[0074] Consequently, as shown in drawing 10 , the band of 7.1kbs equivalent to agdA which Aspergillus oryzae originally has is detected in common. 8.0kbs depending on a high manifestation promotor's die length used in addition to it or the niaD locus in which the band of 8.9kb is detected and a total of two signals exist -- homonous -- 1 -- the stock copied and integrated -- Or the transformation plasmid which was used further in addition to these two signals () [pNAGL136] Or two kinds of patterns of stock ** integrated 2 or more ******s with which the band of 12.0kbs equivalent to the size of pNAGL142 or 12.3kb is also detected, and a total of three signals exist were detected homonous. Moreover, 2 or more ******s of each transformant which was strong as for activity are integrated by plate screening, and it was presumed that the most powerful stock AGL 136-60 of the signal of this inside to 12.0kb was integrated by the most powerful stock AGL 142-72 of the signal of 4 to 5 copies and 12.3kb 4 ******s from 3, respectively.

[0075] Next, in addition to 136 to 60 shares of above AGL(s), 142 to 72 shares of AGL(s), and it, the alpha-glucosidase (AGL) activity of these [which chose at a time two shares of one copy and 2 copy stocks which were obtained from the plasmid used for the transformation, respectively] ten shares of transformants was measured.

[0076] conidium 2 selected platinum loop of ten shares and an old stock niaD14 -- a dextrin peptone (DP) culture medium (2% dextrin --) 1% The poly peptone, 0.5% KH2PO4, 0.05% MgSO4.7H2O or a carbon source -- TSUAPEKKU DOKKUSU poly peptone (CD-P) culture medium of 2% maltose A glucose or 2% (1% poly peptone --) 0.1% K2HPO4, 0.05% MgSO4.7H2O, 0.05% KCl, 0.01% FeSO4 and 2% A carbon source and 15ml of three kinds of

culture media of pH 5.5 were inoculated, and the culture filtrate which removed the fungus body using the glass filter was prepared after shaking culture for 30 degrees C and three days. The alpha-glucosidase (AGL) activity in this culture filtrate In a reaction solution (20mM the acetic-acid buffer solution, pH 5.0, 0.2% 4-nitrophenyl-alpha-D-glucoside (4NPG)), a suitable amount, For example, add 0.1ml culture filtrate and are referred to as 1ml, and add after the time amount (for 1 - 60 minutes) reaction of arbitration, and 0.5M Na2CO3 2.0ml solution at 37 degrees C, and a reaction is suspended. 4-nitrophenol (4NP) which separates in a reaction solution was measured by the rise with an absorbance of 405nm. One unit (U) defined 4NP(s) of 1micromol as the separating activity in 1 minute. Moreover, the quantum of the protein concentration in culture filtrate was carried out with the protein assay stain solution (Bio-Rad make). A measurement result is shown in Table 2.

[0077]

[Table 2]

AGL 導入		AGL活性 (U/mg-protein)			CD-P培地
形質 コピー数					グルコース/マルトース
転換株		DP培地	CD-P培地		(相対活性：%)
			グルコース	マルトース	
136-10	1	1.73	2.69	3.06	88
136-54	1	1.87	2.98	3.42	87
136-32	2	4.13	4.29	5.90	73
136-50	2	3.65	4.91	5.85	84
136-60	4-5	5.21	5.07	6.04	84
142-95	1	2.85	2.87	4.40	65
142-172	1	2.48	3.21	5.00	64
142-107	2	5.51	5.39	6.39	84
142-158	2	5.47	5.33	6.74	79
142-72	3-4	6.15	6.15	7.37	83
niaD14	0	0.065	0.038	0.246	15

[0078] the case where the AGL activity of a transformant is cultivated by which culture medium -- an old stock -- very much -- high -- 1 [for example,] -- it increased 40 or more times by 142 to 95 shares of AGL(s) only by being copied and integrated at the time of DP culture medium. Moreover, also in which [of 136 shares of AGL(s), and 142 shares of AGL(s)] transformant, activity was so high that there were many copy numbers integrated, and the increment in activity was seen [in 136 to 60 shares of AGL(s) which had most copy numbers, and 142 to 72 shares of AGL(s)] 25 or more times by DP culture medium at the time of 130 or more times and a maltose in the time of the glucose of 80 or more times and a CD-P culture medium as a result of Southern analysis. Moreover, P-agdA142 of an amelioration promotor's used activity was higher, and it was [142 shares of AGL(s)] higher in the transformant of the same copy number than P-agdA136 reflecting this. [of activity]

[0079] Since it is integrated homonous when a niaD gene is used for a selective marker, the above result means that it is possible to acquire a transformant with the enzyme activity of arbitration by the copy number integrated with a promotor's strength which is not influenced of the position effect when being included in a chromosome, therefore is used.

Furthermore, AGL by which the relative activity of the glucose to the carbon source maltose of AGL of a transformant shows 70 – 80% on almost all stocks to an old stock showing only 15%, and this is produced by the amelioration promotor means hardly being influenced of a glucose repression.

[0080] Moreover, it checked that performed SDS-polyacrylamide gel electrophoresis and the protein of the same molecular weight as the alpha-glucosidase was increasing clearly about the culture filtrate of these transformants.

[0081]

[Effect of the Invention] A promotor's transcriptional activity not only increases, but by introducing the enhancer DNA sequence of this invention into the promotor who functions with mold, the promotor of the strength of arbitration can be built, amelioration of the manifestation control function is still also attained, and it can improve the existing promotor for a more useful promotor. Moreover, when using the manifestation plasmid including the promotor improved by doing in this way, it became possible on industry to manufacture in large quantities more efficiently by making into a host mold with which safety has high secretion ability for a useful enzyme, protein, a polypeptide, etc. highly. Moreover, the analysis of the promotor about the enhancer sequence of this invention contributes to the elucidation of the manifestation controlling mechanism of mold not a little.

[0082]

[Layout Table]

[0083] array number: -- die-length [of one array]: -- mold [of 3207 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- class [of straight chain-like array]: -- Genomic DNA array: -- TCCGTTTCTT AAAAGAACAA CTACAACACG GTCCGGAATC AACTTGGCGG ACCACGAC 58ATG GCC GGT CTA AAA AGC TTC CTT GCC AGT TCT TGG CTG CTA CCA GTG 106 Met Ala Gly Leu Lys Ser Phe Leu Ala Ser Ser Trp Leu Leu Pro Val 1 5 10 15GCT TGC GGG GCG AGT CAA TCT ATC GTT CCT AGC ACT TCG GCA ACA GCG 154 Ala Cys Gly Ala Ser Gln Ser Ile Val Pro Ser Thr Ser Ala Thr Ala 20 25 30GCA TAC TCG CAG TTC ACC ATT CCC GCC TCT GCC GAT GTG GGC GCG AAT 202 Ala Tyr Ser Gln Phe Thr Ile Pro Ala Ser Ala Asp Val Gly Ala Asn 35 40 45TTG GTC GCCAAC ATT GAT GACCCC CAA GCG GTC AAC GCG CAA TCT GTC 250 Leu Val Ala Asn IleAsp Asp Pro Gln Ala Val Asn Ala Gln Ser Val 50 55 60TGT CCG GGC TAC AAG GCC TCC GATGTG AAA CAT TCC TCC CAG GGT TTC 298 CysPro Gly Tyr Lys Ala Ser Asp Val Lys His Ser Ser Gln Gly Phe 65 70 75 80ACC GCT AGC CTG GAG TTG GCT GGA GAC CCT TGT AAT GTT TAC GGA ACG 346 Thr AlaSer Leu Glu Leu Ala Gly Asp Pro Cys Asn Val Tyr Gly Thr 85 90 95 GAC GTC GAT TCG TTGACC CTG ACC GTG GAA TAC CAG GCA AAG GAT CGT 394 Asp Val AspSer Leu Thr Leu Thr Val Glu Tyr Gln Ala Lys Asp Arg 100 105 110TTG AAC ATC CAG ATT GTT CCG ACG TAT TTT GAC GCC TCC AAT GCA TCT 442 Leu Asn Ile Gln Ile Val Pro Thr Tyr Phe Asp Ala Ser Asn Ala Ser 115 120 125TGG TAC ATTCTT TCG GAA GAGCTA GTG CCC AGA CCA AAG GCT TCC CAA 490 Trp Tyr Ile Leu Ser Glu Glu Leu Val Pro Arg Pro Lys Ala Ser Gln 130 135 140AAT GCA TCG GTT CCT CAG AGT GAT TTT GTT GTC TCT TGG TCC AAC GAA 538 Asn Ala Ser Val Pro GlnSer Asp Phe Val Val Ser Trp Ser Asn Glu145 150 155 160CCT TCT TTC AACTTT AAG GTG ATC CGA AAA GCT ACT GGT GAC GTG CTA 586 Pro SerPhe Asn Phe Lys Val Ile Arg Lys Ala Thr Gly Asp Val Leu 165 170 175TTC AAC ACA AAG GGC TCT ACC TTA GTC TAC GAG AAT CAG TTC ATA GAA 634 Phe Asn Thr Lys Gly Ser Thr Leu Val Tyr Glu Asn Gln Phe Ile Glu 180 185 190TTT GTC ACG TTG TTG CCT-GAA-GAA-TAT-AAC CTA TAT GGC TTG GGA GAG 682Phe Val Thr-Leu-Leu-Pro-Glu Glu Tyr Asn Leu Tyr-Gly-Leu-Gly-Glu 195 200 205CGG ATG AAC CAG CTG CGG

CTA CTG GAG AAC GCT AAT TTG ACG CTA-TAT730 Arg Me sn Gln Leu Arg Leu Leu Glu Asn Ala Asn Leu Thr Leu Tyr 210 215 220 GCC GCA GAT ATC GCA GAT CCC ATT GAC GA gtacgtatct ggcatttggt gc 781 Ala Ala Asp Ile Ala Asp Pro Ile Asp As225 230 ggcccatt aggttttagg ctaatccatg gacctctag T AAC ATC TAT GGA CAT CA 837 p Asn Ile Tyr GlyHisHis 235 240GCA TTT TAC TTG GAT ACA AGG TAC TAC AAG GTG GGT GGT CAG AAT AAG 885 Ala Phe Tyr Leu Asp Thr Arg Tyr Tyr Lys Val Gly Gly Gln Asn Lys 245 250 255AGC CATACC ATA GTC AAG AGC AGCGAA GCG GAA CCA TCT CAA GAA TAC 933 Ser His Thr Ile Val Lys Ser Ser Glu Ala Glu Pro Ser Gln Glu Tyr 260 265 270GTC TCA TAT TCT CAC GGA GTG TTC CTC AGA AAT GCC CAT GGA CAG GAG 981 Val Ser Tyr Ser His Gly Val Phe Leu Arg Asn Ala His Gly Gln Glu 275 280 285ATC CTC CTGCGG GAT CAA AAGTTG ATC TGG CGC ACT CTG GGA GGA AGC 1029 Ile Leu Leu Arg AspGln Lys Leu Ile Trp Arg Thr Leu Gly Gly Ser 290 295 300GTT GAT CTG ACA TTC TAC TCT GGC CCA ACG CAA GCC GAG GTC ACC AAG 1077 Val Asp Leu Thr Phe TyrSer Gly Pro Thr Gln Ala Glu Val Thr Lys305 310 315 320CAA TAT CAG CTCAGC ACC GTG GGA CTG CCT GCC ATG CAG CAA TAT AAC 1125 Gln TyrGln Leu Ser Thr Val Gly Leu Pro Ala Met Gln Gln Tyr Asn 325 330 335ACG CTC GGA TTT CAC CAG TGC CGC TGG GGC TAT AAC AAC TGG TCC GAA 1173 Thr Leu Gly Phe His Gln Cys Arg Trp Gly Tyr Asn Asn Trp Ser Glu 340 345 350TTTGAA GAC GTA CTT GCCAAT TTC GAG AGA TTC GAG ATT CCT TTG GAG 1221 Phe Glu Asp ValLeu Ala Asn Phe Glu Arg Phe Glu Ile Pro Leu Glu 355 360 365 TAC CTC TG gtaagaaaca tgttcgtcgc tcattcggct tccttctaac gcctatat 1277Tyr Leu Tr 370 gcag G GCC GAT ATC GAT TAC ATG CAT GGA TAT CGC AAT TTT GAC 1321 p Ala Asp Ile Asp Tyr Met His Gly Tyr Arg Asn PheAsp 375380 AAT-GAC-CAA-CAT-CGC TTT-TCG-TAT-GAA-GAA GGT GAA AAG TTC CTC AAC 1369Asn Asp Gln-His-Arg-Phe-Ser Tyr Glu Glu Gly Glu-Lys-Phe-Leu-Asn385 390 395 400AAG CTT CAC GCC GGT GGA CGT CGC TGG GTC CCA ATC GTT GAC GGA GCT 1417 Lys Leu His Ala Gly Gly Arg Arg Trp Val Pro Ile Val Asp Gly Ala 405 410 415 CTT TAT ATT CCCAATCCG GAG AAC GCT TCT GAT GC gtaagtggccgtctt 1467 Leu Tyr Ile Pro Asn Pro Glu Asn Ala Ser Asp aluminum 420 425 ccaca tactcttgcc cgtgaacgaa gactcaccgt gattatag T TAC GAA ACT TAT 1523 a Tyr Glu Thr Tyr 430GAC AGA GGC GCC AAG GAC GAT GTT TTC ATC AAG AAT CCC GAC GGC AGT 1571 Asp Arg Gly Ala Lys Asp Asp Val Phe Ile LysAsn Pro Asp GlySer 435 440 445CTA TAC ATT GGC GCT GTC TGG CCT GGC TAT ACT GTC TAC CCC GACTGG 1619 Leu Tyr Ile Gly Ala Val Trp Pro Gly Tyr Thr Val Tyr Pro Asp Trp 450 455 460CAT CAT CCT AAG GCC TCC GAT TTC TGG GCT AAT GAG CTGGTC ACC TGG 1667 His His Pro Lys Ala SerAsp Phe Trp Ala Asn Glu Leu Val Thr Trp465 470 475 480TGG AAC AAG CTG CAT TAT GAT GGG GTC TGG TAC GAC ATG GCT GAA GTT 1715 Trp AsnLys Leu His Tyr Asp Gly Val Trp Tyr Asp Met Ala Glu Val 485 490 495 TCT TCC TTC TGC GTAGGG AGC TGC GGA ACT GGC AAT CTG TCA ATG AAC 1763 Ser Ser PheCys Val Gly Ser Cys Gly Thr Gly Asn Leu Ser Met Asn 500 505 510CCG GCT CAT CCA CCG TTC GCT CTC CCC GGC GAA CCA GGG AAC GTC GTC 1811 Pro Ala His Pro Pro Phe Ala Leu Pro Gly Glu Pro Gly Asn Val Val 515 520 525TAT GAT TATCCA GAG GGC TTTAAC ATC ACC AAT GCT ACG GAA GCA GCC 1859 Tyr Asp Tyr Pro GluGly Phe Asn Ile Thr Asn Ala Thr Glu Ala Ala 530 535 540TCA GCA TCC GCT GGG GCG GCA AGC CAA TCC GCA GCG GCA TCA TCC ACA 1907 Ser Ala Ser Ala Gly AlaAla Ser Gln Ser Ala Ala Ala Ser Ser Thr545 550 555 560ACT ACA TCA GCCCCC TAC CTG CGT ACA ACA CCT ACC CCC GGA GTT CGT 1955 Thr ThrSer Ala Pro Tyr Leu Arg Thr Thr Pro Thr Pro Gly Val Arg 565 570 575 AAT GTT GAC CAC CCT CCT TAT GTT ATC AAC CAT GTC CAA CCT GGC CAC 2003Asn Val Asp His Pro Pro-Tyr-Val-Ile-Asn His Val Gln Pro Gly-His 580 585 590GAC CTG AGC GTT CAC GCC-ATC-TCA-CCA-AAT TCT ACT CAC TCG GAT

GGG 2051Asp Leu Ser-Val-His-Ala-Ile Ser Pro Asn Ser Thr-His-Ser-Asp-Gly 595 600 605GTC CAG GAG TAT GAT GTA CAC AGT CTTTAC GGC CAC CAA GGC ATA AAT 2099 Val Gln Glu Tyr Asp Val HisSer Leu Tyr Gly His Gln Gly Ile Asn 610 615 620GCA ACC TAT CAC GGA TTG CTC AAG GTG TGG GAG AAC AAA CGC CCC TTT 2147 Ala Thr Tyr His Gly LeuLeu Lys Val Trp Glu Asn Lys Arg Pro Phe625 630 635 640ATC ATC GCA CGCTCT ACA TTT TCC GGC TCT GGG AAA TGG GCC GGC CAC 2195 Ile IleAla Arg Ser Thr Phe Ser Gly Ser Gly Lys Trp Ala Gly His 645 650 655TGG GGT GGT GAT AAC TTC TCC AAA TGG GGA TCG ATG TTC TTT TCG ATC 2243 Trp Gly Gly Asp Asn Phe Ser Lys Trp Gly Ser Met Phe Phe Ser Ile 660 665 670TCGCAG GCC CTC CAG TTCTCG CTC TTT GGC ATC CCT ATG TTT GGT GTT 2291 Ser Gln Ala LeuGln Phe Ser Leu Phe Gly Ile Pro Met Phe Gly Val 675 680 685GAC ACC TGT GGT TTC AAT GGA AAC ACG GAT GAG GAG CTA TGC AAC CGA 2339 Asp Thr Cys Gly PheAsn Gly Asn Thr Asp Glu Glu Leu Cys Asn Arg 690 695 700TGG ATG CAG CTC TCG GCC TTT TTCCCT TTC TAC CGC AAC CAT AAT GTT 2387 Trp Met Gln Leu Ser AlaPhe Phe Pro Phe Tyr Arg Asn His Asn Val705 710 715 720CTC TCT GCA ATC CCA CAA GAG CCC TAT CGG TGG GCG TCC GTG ATC GAT 2435 Leu SerAla Ile Pro Gln Glu Pro Tyr Arg Trp Ala Ser Val Ile Asp 725 730 735 GCC ACG AAG GCG GCAATG AAC ATT CGA TAC GCT ATT TTG CCG TAC TTT 2483 Ala Thr LysAla Ala Met Asn Ile Arg Tyr Ala Ile Leu Pro Tyr Phe 740 745 750TAC ACC CTG TTC CAT TTG GCC CAC ACC ACT GGA TCT ACG GTC ATG CGC 2531 Tyr Thr Leu Phe His Leu Ala His Thr Thr Gly Ser Thr Val Met Arg 755 760 765GCA CTT GCGTGG GAG TTC CCGAAT GAC CCC TCC CTA GCT GCT GTC GGC 2579 Ala Leu Ala Trp GluPhe Pro Asn Asp Pro Ser Leu Ala Ala Val Gly 770 775 780ACC CAA-TTT-CTT-GTC-GGT CCC-TCG-GTC-ATG-GTG ATT CCT GTT CTT GAG 2627Thr Gln Phe Leu-Val-Gly-Pro-Ser Val Met Val Ile Pro-Val-Leu-Glu785 790 795 800CCA CAG GTA GAT ACT GTC CAG GGT GTC TTC CCA GGT GTT GGA CAT GGG 2675 Pro Gln Val Asp Thr Val Gln Gly Val Phe Pro Gly Val Gly His Gly 805 810 815 GAA GTC TGG TACGACTGG TAC TCT CAA ACA GCT GTT GAT GCA AAG CCC 2723 Glu Val TrpTyr Asp Trp Tyr Ser Gln Thr Ala Val Asp Ala Lys Pro 820 825 830GGT GTC AAC ACA ACA ATC TCA GCG CCA CTG GGC CAC ATT CCG GTT TTC 2771 Gly Val Asn Thr Thr Ile Ser Ala Pro Leu Gly His Ile Pro Val Phe 835 840 845GTT CGT GGTGGT AGC ATT CTGCCC ATG CAG GAG GTT GCG CTG ACC ACT 2819 Val Arg Gly Gly SerIle Leu Pro Met Gln Glu Val Ala Leu Thr Thr 850 855 860CGC GAC GCT CGC AAG ACC CCC TGG TCT TTG CTC GCG TCG CTG AGC AGT 2867 Arg Asp Ala Arg Lys ThrPro Trp Ser Leu Leu Ala Ser Leu Ser Ser865 870 875 880AAT GGA ACT GCCTCT GGC CAG CTC TAC CTC GAT GAT GGA GAA AGT GTC 2915 Asn GlyThr Ala Ser Gly Gln Leu Tyr Leu Asp Asp Gly Glu Ser Val 885 890 895TAC CCC GAG GAT ACG CTT TCT GTG GAC TTC CTG GCG TCT CGC TCC ACT 2963 Tyr Pro Glu Asp Thr Leu Ser Val Asp Phe Leu Ala Ser Arg Ser Thr 900 905 910CTCCGA GCC TCT GCG CGGGGT ACT TGG AAG GAG GCG AAT CCA CTA GCG 3011 Leu Arg Ala SerAla Arg Gly Thr Trp Lys Glu Ala Asn Pro Leu Ala 915 920 925AAT GTG ACG GTA CTT GGT GTG ACT GAG AAG CCA TCC TCA GTG ACA CTC 3059 Asn Val Thr Val LeuGly Val Thr Glu Lys Pro Ser Ser Val Thr Leu 930 935 940AAT GGC GAG ACG CTC TCC TCC GACTCT GTG AAG TAT AAC GCC ACC TCA 3107 Asn Gly Glu Thr Leu SerSer Asp Ser Val Lys Tyr Asn Ala Thr Ser945 950 955 960CAC GTT CTC CAC GTT GGT GGC TTG CAG AAG CAC ACA GCG GAT GGA GCA 3155 His ValLeu His Val Gly Gly Leu Gln Lys His Thr Ala Asp Gly Ala 965 970 975 TGG GCG AAG GAC TGGGTA CTG AAA TGG TGAAGGAAGC GTAACAGGATAGCCT 3207 Trp Ala Lys Asp Trp Val Leu Lys Trp 980 985 [0084] array number: -- die-length [of two arrays]: -- mold [of 720 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- class [of straight chain-like array]: -- Genomic DNA Array :

GCCATCGGAT GCTCCCGTGA TGGCACCACT AGAGATGGCG GAAAACCC
TCACCGGCAC 60 ACCGGAGGGG TTTAGGCACC TTGGAATATG AGGTGGGGAA
CGATGTATTT GCCAGTATTG 120 ACTCTGGTGA ATGGATCTCT CGAGAAATAC
TACCTTTTCA GGGCTCAAGC GTCGTGTCGG 180 GCATTTATCG GGGGATGGAC
CAATCAGCGT AGGGATATCA GATGATCGCC AGCATTGGTC 240 AGGAACGTTT
CCAATTTCCG GACACGGAAG TACTGTAACT GCTCCCAAGA ATCAACACAC 300
TCTTTTCCGG TCTCGTCCTT TGCTCGGCAG AGATTCATCT CCCATCGTCG
GCTTAACCGG 360 TACTCTTTCG TCACGTTCCA AAAGGCTTGA TCATGCTGTC
CCCACTCCGT GCGGGTGAAG 420 CCACCTCATT GCTGCGTAGG ACCTATACCC
TTCAACTAGC GTGACTTCTT CCCCTCTCAT 480 GGTCGAGAGA TTGCAGGCAA
TGCCCCTCGG ACGTTTGACG GGGAATGTTT TGCCTTCACG 540 GCAGGTAGCA
CAAATCGATG GGAACGGGAC GGGCCATCAA TTGTGAGGGA TTTCCCGTGG 600
ACACCTGGTT CGTCAAGACA TATACATCTA GCTACAATTC CGGTTCGGAG
ACGGCAGAGG 660 GGTCCGTTTC TTAAAAGAAC AACTACAACA CGGTCCGGAA
TCAACTTGGC GGACCACGAC 720 [0085] array number: -- die-length [of three arrays]: -- mold [of 840 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- class [of straight chain-like array]: -- Genomic DNA array: -- TGAAGGAAGC
GTAACAGGAT AGCCTAGACC CACATACTAT CTGTATACAA CTCCGCAATA 60
TGAAGTGATG AATGCAAACT AGCAGCGAAT CGGATATCAG TAGCATAACG
TAATCGGTAA 120 GCGAGTTGCC CGCGCAAGCG AGTTGCCCAC CACACGCGTT
TTCAACGCGC CTCAATTTCT 180 TAGATGATTA AAACATCAGC CATACCACCA
AAAATACCTA AATCAAAAAA ATCACGCGTT 240 GAGCAGGAAG GCAGAATCCT
ATTAGCTATA TCAGCTATAA AAAAACAAGA AATCAGCAGT 300 TTTAGAAAGG
CAGCTGAAAT TTTTAATATA CCTATCGCTA CACTACGTTA TCGTCTAAAT 360
GGAGGTTCCT TTCGAAATGA TACTCGTGCC AATAGTTATA AAATAACTTC
TAGTGAAGAG 420 AAATCGCTTA AAAAATAGAT TCTATCACTA GATAAACGTG
GAGCACCTCC TCGGCCTGTA 480 CACGTACGAG AAATAGCCAA TATCCTGCTT
TTAAAGCGTA ATACTACCTC CCCCCCTACT 540 ACTGTAGAGG AGAAATGGGT
ATACAACTTT ACCAAGCGTA CACCTAAGCT TAAATTCTGC 600 TTTGCACGTC
GCTACAACTA TCAGCATGCC AAGGTAGAGG ATCCTAAGGT TCTAGGTACT 660
TAGTTTAAGC AGGTAAATAA GGTTATTCAG AAGTACGGTA TAGCTTCAAG
CGATATATAC 720 AATTTTAATA AAACGGGGTT TATAATGGGC CTAATAGCTA
CAGCCAAAGT TGTTACTAGA 780 TCTAATATGC CAGGGAAACT ATTTTTATTA
CAGCTAGAGA ACCGGGAATG GGTTACTGCC 840

[Translation done.]

* NOTICES *

DESCRIPTION OF DRAWINGS

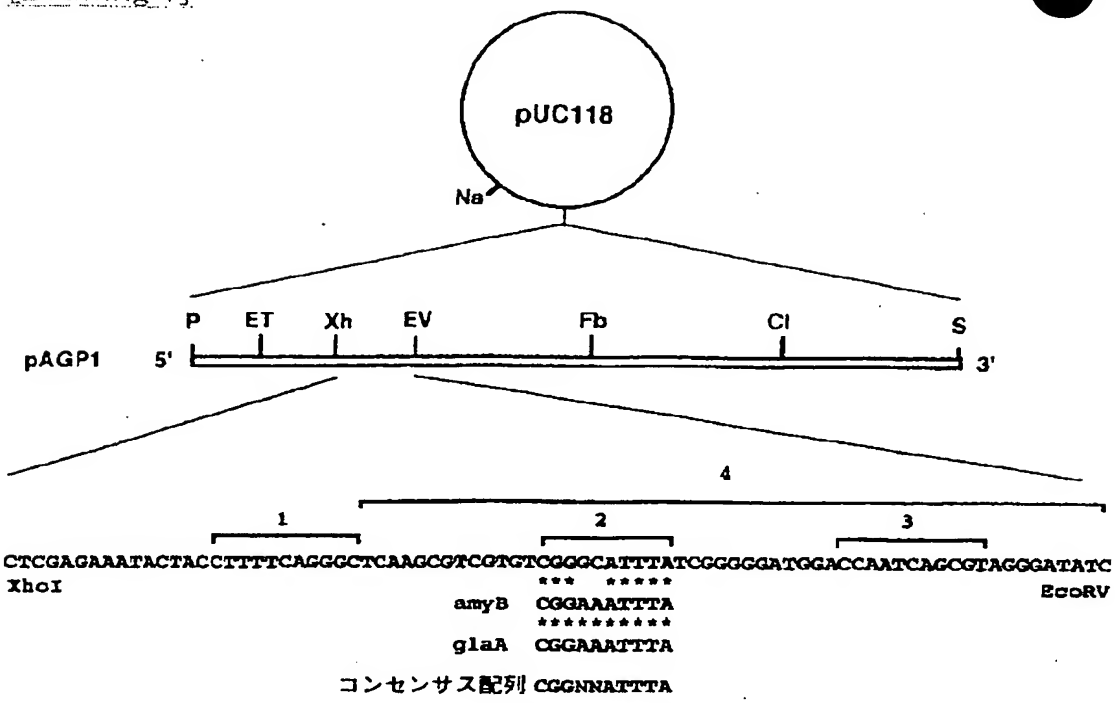
[Brief Description of the Drawings]
[Drawing 1] The restriction enzyme map of the promoterregion of an alpha-glucosidase gene, and an enhancer sequence and the base sequence of the circumference of it are shown.
[Drawing 2] A plasmid pNAGT4 and the construction procedure of pNAGG 1-1 are shown.
[Drawing 3] The homonous integration pattern in the niaD locus of a GUS gene is shown.
[Drawing 4] The relative activity of the various deletion promotors of an alpha-glucosidase gene is shown.
[Drawing 5] The promotor activity when permuting two base pairs of the fluctuation part of the enhancer base sequence B by the base of arbitration is shown.
[Drawing 6] The promotor activity when introducing the enhancer base sequence E into a promotor is shown.
[Drawing 7] It is the drawing substitution photograph of electrophoresis in which the northern blot analysis of the promotor who introduced the enhancer base sequence E is shown.
[Drawing 8] The construction procedure of plasmids pNAG136 and pNAG142 is shown.
[Drawing 9] The construction procedure of plasmids pNAGL136 and pNAGL142 is shown.
[Drawing 10] The homonous integration pattern in the niaD locus of an alpha-glucosidase gene is shown.

[Translation done.]

* NOTICES *

DRAWINGS

[Drawing 1]



Na : NaeI, P : PstI, ET : EcoT14I, Xh : XhoI, EV : EcoRV, Fb : FbaI , Cl : ClaI , S : SalI

amyB : α－アミラーゼ遺伝子 プロモーターの高ホモロジー配列

glaA ：グルコアミラーゼ遺伝子 プロモーターの高ホモロジー配列

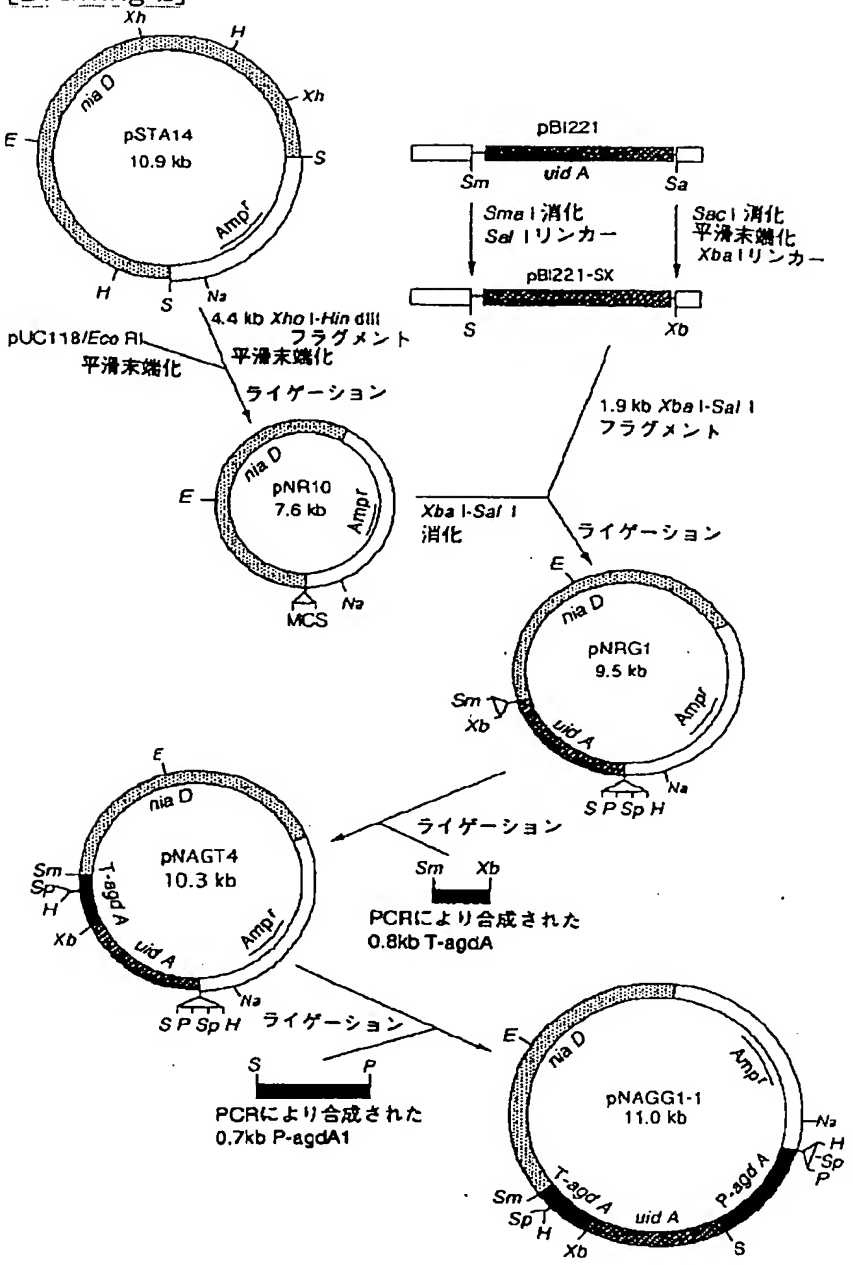
1 : P-agdA8で欠失している塩基配列

2 : P-agdA6で欠失している塩基配列（エンハンサー配列Ｂ）

3 : P-agdA7で欠失している塩基配列（エンハンサー配列Ｃ）

4 : エンハンサー配列Ｂ及びＣを含むＤＮＡ塩基配列単位Ｅ

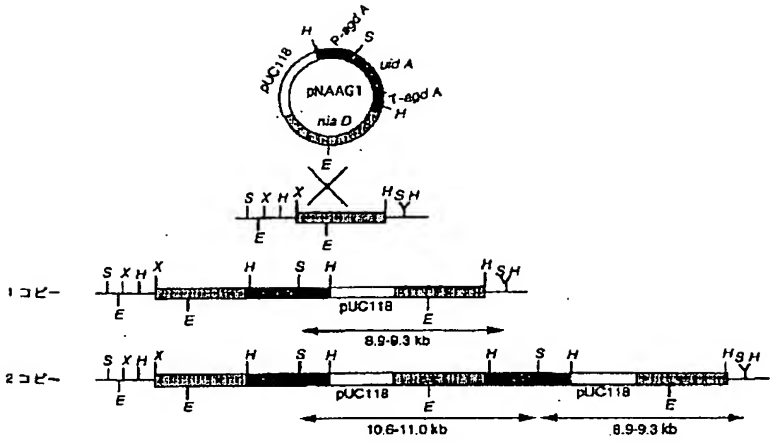
[Drawing 2]



Xh: XhoI, H: HindIII, S: SalI, Na: NaeI, Sm: SmaI, Sa: SacI, Xb: XbaI, E: EcoRI, P: PstI, Sp: SphI,

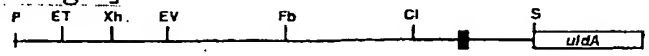
P-agdA : α-グルコシダーゼ遺伝子プロモーター
T-agdA : α-グルコシダーゼ遺伝子ターミネーター
niaD : 硝酸還元酵素遺伝子（選択マーカー）
uidA : β-グルクロニダーゼ遺伝子(レポーター遺伝子)
Amp : アンピシリン耐性遺伝子
MCS : マルチクローニングサイト

[Drawing 3]



S: SalI, E: EcoRI, X: XhoI, H: HindIII
P-agdA : α-グルコシダーゼ遺伝子プロモーター
T-agdA : α-グルコシダーゼ遺伝子ターミネーター
niaD : 硝酸還元酵素遺伝子（選択マーカー）
uidA : β-グルクロニダーゼ遺伝子（レポーター遺伝子）

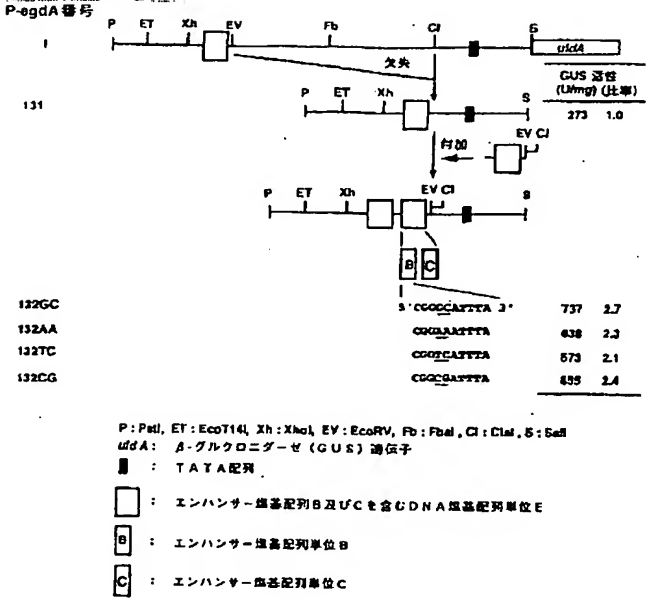
[Drawing 4]



P-agdA 番号	GUS 活性 (%)
1	100
2	25.5
3	28.7
4	6.4
5	6.1
6	7.0
7	19.0
8	75.2

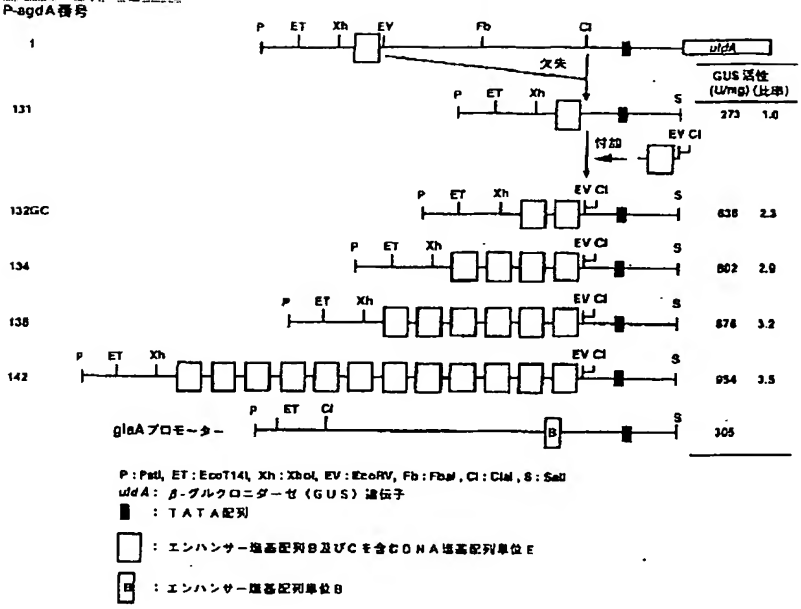
P : PstI, ET : EcoT14I, Xh : XhoI, EV : EcoRV, Fb : FbaI , Cl : ClaI , S : SalI
uidA : β-グルクロニダーゼ（ＧＵＳ）遺伝子
■ : ＴＡＴＡ配列

[Drawing 5]

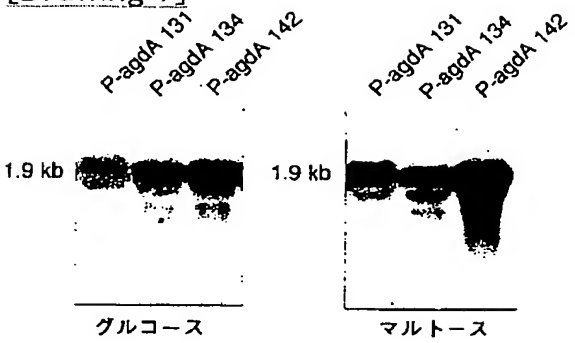


P : PstI, ET : EcoT14I, Xh : XhoI, EV : EcoRV, Fb : FbaI, Cl : ClaI, S : SalI
uidA : β-グルクロニダーゼ（ＧＵＳ）遺伝子
▮ : ＴＡＴＡ配列
□ : エンハンサー塩基配列Ｂ及びＣを含むＤＮＡ塩基配列単位Ｅ
Ⓑ : エンハンサー塩基配列単位Ｂ
Ⓒ : エンハンサー塩基配列単位Ｃ

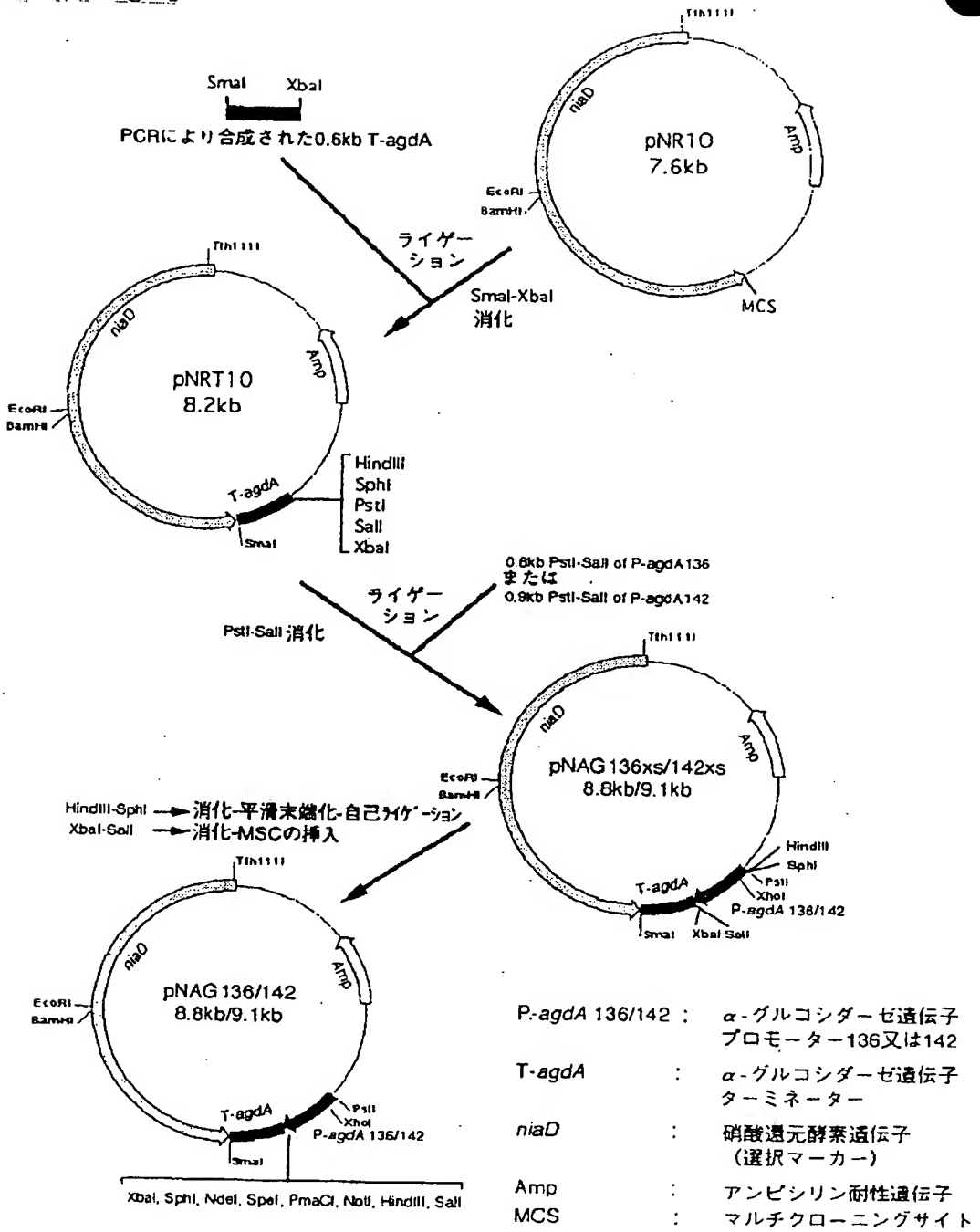
[Drawing 6]


P-agdA番号
GUS活性 (U/mg) (比率)
1
欠失
273 1.0
131
付加
132GC
638 2.3
134
802 2.9
138
878 3.2
142
954 3.5
gleAプロモーター
305
P : PstI, ET : EcoT14I, Xh : XhoI, EV : EcoRV, Fb : FbaI , Cl : ClaI , S : SalI
uidA : β-グルクロニダーゼ（GUS）遺伝子
：ＴＡＴＡ配列
：エンハンサー塩基配列Ｂ及びＣを含むＤＮＡ塩基配列単位Ｅ
：エンハンサー塩基配列単位Ｂ

[Drawing 7]

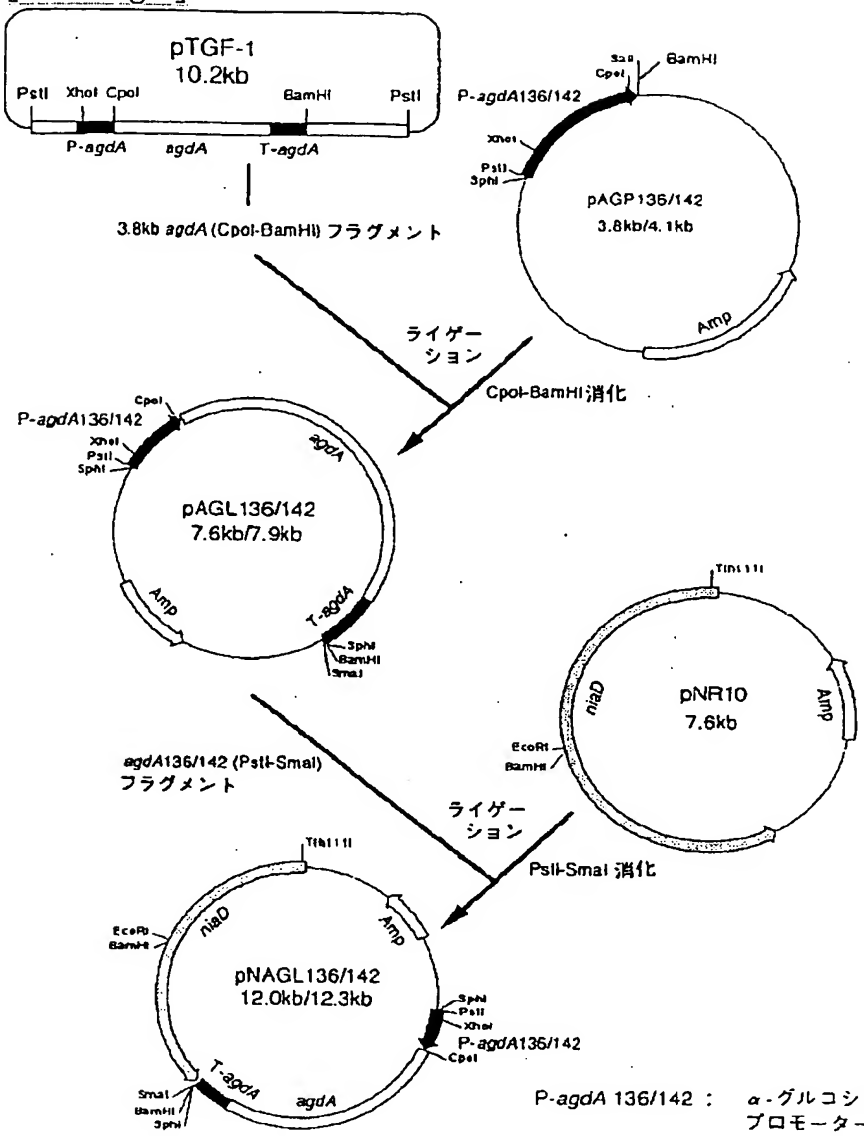

P-agdA 131
P-agdA 134
P-agdA 142
1.9 kb
グルコース
P-agdA 131
P-agdA 134
P-agdA 142
1.9 kb
マルトース

[Drawing 8]



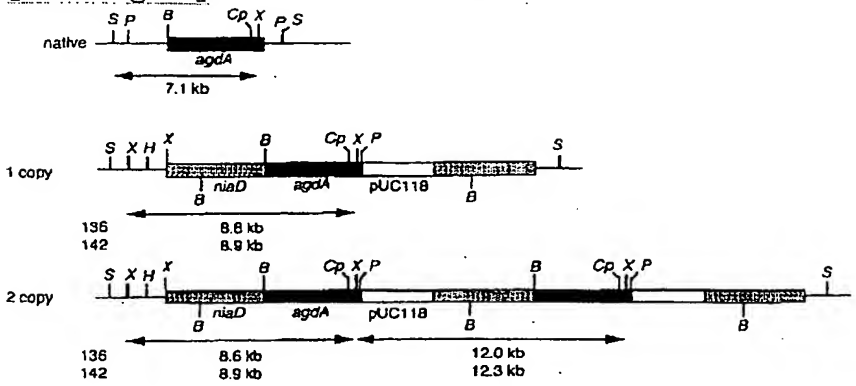
P-agdA 136/142	:	α-グルコシダーゼ遺伝子プロモーター136又は142
T-agdA	:	α-グルコシダーゼ遺伝子ターミネーター
niaD	:	硝酸還元酵素遺伝子（選択マーカー）
Amp	:	アンピシリン耐性遺伝子
MCS	:	マルチクローニングサイト

[Drawing 9]



P-*agdA* 136/142	:	α-グルコシダーゼ遺伝子プロモーター136又は142
T-*agdA*	:	α-グルコシダーゼ遺伝子ターミネーター
agdA	:	α-グルコシダーゼ構造遺伝子
niaD	:	硝酸還元酵素遺伝子（選択マーカー）
Amp	:	アンピシリン耐性遺伝子

[Drawing 10]



S: SalI, X: XhoI, H: HindIII, B: BamHI, Cp: CpoI, P: PstI

niaD : 硝酸還元酵素遺伝子（選択マーカー）

agdA : α-グルコシダーゼ遺伝子

[Translation done.]

（書誌＋要約＋請求の範囲）

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(51)【国際特許分類第7版】

```
   C12N 15/09    ZNA
         1/15
//(C12N 15/09    ZNA
   C12R  1:66   )
  (C12N  1/15
   C12R  1:66   )
```

【FI】

```
   C12N 15/00    ZNA A
         1/15
```

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【請求項の数】5
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【識別番号】100074206
【弁理士】
【氏名又は名称】鎌田 文二 (外2名)

(57)【要約】
【課題】アスペルギルス属糸状菌の遺伝子工学において使用可能な選択マーカーとして機能するピリチアミン耐性マーカー遺伝子を提供し、また、遺伝学的解析に役立つ表現型に形質が転換された形質転換体を提供することである。
【解決手段】以下の(a)または(b)のDNAからなるピリチアミン耐性マーカー遺伝子とする。
(a)配列表の配列番号1で表される塩基配列からなるDNA。
(b)塩基配列(a)において1もしくは数個の塩基が置換、欠失または付加された塩基配列からなり、かつDNA(a)と同様のピリチアミン耐性を発現するピリチアミン耐性マーカー遺伝子。
上記のピリチアミン耐性マーカー遺伝子で形質転換され、ピリチアミン耐性を発現するアスペルギルス(aspergillus)属糸状菌などの形質転換体とする。

【特許請求の範囲】
【請求項1】以下の(a)または(b)のDNAからなるピリチアミン耐性マーカー遺伝子。
(a)配列表の配列番号1で表される塩基配列からなるDNA。
(b)塩基配列(a)において1もしくは数個の塩基が置換、欠失または付加された塩基配列からなり、かつDNA(a)と同様のピリチアミン耐性を発現するピリチアミン耐性マーカー遺伝子。
【請求項2】請求項1記載のピリチアミン耐性マーカー遺伝子で形質転換され、ピリチアミン耐性を発現する形質転換体。
【請求項3】ピリチアミン耐性マーカー遺伝子を導入して形質転換される宿主が、アスペルギルス(Aspergillus)属糸状菌である請求項2記載の形質転換体。
【請求項4】下記工程を包含することを特徴とするピリチアミン耐性形質転換体の創製方法。
(1)請求項1記載のピリチアミン耐性マーカー遺伝子を含有するベクターを複製する工程、(2)上記工程で得られた複製ベクターを宿主の染色体に組み込む工程、(3)上記工程でピリチアミン耐性に形質転換された宿主をピリチアミン存在下で選択する工程。
【請求項5】複製ベクターが、請求項1記載のピリチアミン耐性マーカー遺伝子以外の異種の遺伝子を含有する複製ベクターである請求項4記載の形質転換体の創製方法。

詳細な説明

【発明の詳細な説明】
【0001】
【発明の属する技術分野】この発明は新規な薬剤耐性遺伝子からなるピリチアミン耐性マーカー遺伝子およびこの遺伝子を用いたアスペルギルス属糸状菌などを宿主とする形質転換体およびその創製方法に関する。
【0002】
【従来の技術】アスペルギルス属糸状菌を主とする麹菌は清酒、焼酎、醤油の生産等の発酵食品または各種有用物質の生産等に広範に利用されている。これらの工業用に使用されるアスペルギルス属糸状菌の育種において、望ましい形質を付与するために遺伝子工学的手法はきわめて有効であり、形質転換体を効率よく選択するために使用可能な選択マーカーが必要である。
【0003】糸状菌において形質転換体の優性マーカーとなりえる薬剤耐性遺伝子としては、ペニシリウム ロックフォルテイー(Penicillium roqueforti)のphelomycin耐性遺伝子(J. Biotccol 1996 0ct. 18;51(1):97－105)、ペニシリウムアイスランジューム(Penicillium islandicum)のbenomyl 耐性遺伝子(curr. Genet. 1995 Nov.;28(6)580-4)、アスペルギルス ニガー(Aspergillusniger 以下 A. ニガーと略記する。)のhygromycin B耐性遺伝子(Gene. 1987;56(1):117-24)やoligomycin耐性遺伝子(Curr. Genet.1988 Jul;14(1):37-42)、アスペルギルス ニドランス(Aspergillus nidulans 以下 A. ニドランスと略記する。)のオーレオバシジン A 耐性遺伝子(特開平9-98784 号公報)または、アスペルギルス フラバス(Aspergillus flavus)のベノミル耐性遺伝子(Appl. Environ Microbiol 1990 Dec;56(12):3686-92)等が挙げられる。
【0004】現在、麹菌 A. オリーゼにおける遺伝子組み換えにおいては栄養要求性を相補する遺伝子を選択マーカーとして利用しており、例としてはargB(Agric. Biol.Chem. 51(9), 2549－2555, 1987)、またはniaD(Gene 111(2),149－55, 1992Feb 15)が挙げられる。
【0005】
【発明が解決しようとする課題】しかし、栄養要求性を相補する選択マーカーを用いるためには、紫外線照射、変異源物質等による突然変異誘導により栄養要求性株を作製する必要がある。そして、この無作為な突然変異誘導によれば、宿主株の性質に何らかの影響を及ぼす可能性が高い。
【0006】また、清酒、醤油、みそ、又はみりん等の各醸造産業で利用される重要な麹菌、アスペルギルス オリーゼ(Aspergillus oryzae 以下A.オリーゼと略記する。)は広範な薬剤に対して強い抵抗性を示すことから優性マーカーとして有効な薬剤耐性遺伝子は未だ発見されていない。
【0007】以上の理由から A. オリーゼに対し、その性質に影響を及ぼすことなく遺伝子組み換えを行うことができる薬剤耐性優性選択マーカーが要望されていた。
【0008】そこで、この発明の課題は、アスペルギルス属糸状菌の遺伝子工学的使用において選択マーカーとして適用できるピリチアミン耐性遺伝子を提供する事である。また、この発明は、このような薬剤耐性遺伝子を含有するベクターによって遺伝学的解析に役立つ表現型に形質が転換された形質転換体を提供することをも課題としている。
【0009】
【課題を解決するための手段】本願の発明者らは、所定のアスペルギルス属糸状菌が、チアミンの代謝括抗アナログであるピリチアミンに対して鋭敏な感受性を示すことを見いだし、その感受性細胞に変異処理を施すことにより耐性細胞に変え、その耐性細胞よりA.オリーゼに対してピリチアミン耐性を付与する遺伝子(耐性遺伝子)を単離することに成功し、この発明を完成した。
【0010】すなわち、本願の第1の発明においては、以下の(a)または(b)のDNAからなるピリチアミン耐性マーカー遺伝子とする手段を採用することにより、前述の課題を解決したのである。
(a)配列表の配列番号1で表される塩基配列からなるDNA。
(b)塩基配列(a)において1もしくは数個の塩基が置換、欠失または付加された塩基配列からなり、かつDNA(a)と同様のピリチアミン耐性を発現するピリチアミン耐性マーカー遺伝子。
【0011】また、本願の第2の発明では、上記の第1の発明のピリチアミン耐性マーカー遺伝子で形質転換され、ピリチアミン耐性を発現する形質転換体とすることにより、前述の課題を解決したのである。また、上記の発明において、ピリチアミン耐性マーカー遺伝子を導入して形質転換される宿主が、アスペルギルス(Aspergillus)属糸状菌である形質転換体とした場合にも前述の課題を解決することができる。
【0012】また、本願の第3の発明においては、下記の工程を包含することを特徴とするピリチアミン耐性形質転換体の創製方法としたのである。
(1)請求項1記載のピリチアミン耐性マーカー遺伝子を含有するベクターを複製する工程、(2)上記工程で得られた複製ベクターを宿主の染色体に組み込む工程、(3)上記工程でピリチアミン耐性に形質転換された

宿主をピリチアミン存在下で選択する工程。
【0013】このピリチアミン耐性形質転換体の創製方法において複製ベクターは、配列番号1に記載されたピリチアミン耐性マーカー遺伝子以外の異種の遺伝子を含有する複製ベクターであってもよい。
【0014】この発明のピリチアミン耐性を付与する遺伝子、すなわちピリチアミン耐性マーカー遺伝子は、アスペルギルス属糸状菌の細胞の染色体に組み込まれた際にピリチアミン耐性を発現する。また、ピリチアミン耐性マーカー遺伝子を組み込まれたアスペルギルス属糸状菌を特異的培地内に置くことによって、これらの細胞の中からピリチアミン耐性のある形質転換体を容易に検出することができる。
【0015】このようなピリチアミン耐性マーカー遺伝子は、遺伝学的解析に役立つ表現型のはっきりした遺伝子であり、ピリチアミン耐性のある形質転換体についても遺伝学的解析に役立つものである。
【0016】
【発明の実施の形態】この発明のピリチアミン耐性マーカー遺伝子は、前述のようにチアミンの代謝括抗アナログであるピリチアミンに対して鋭敏な感受性を示すピリチアミン感受性細胞に対して、変異処理を施すことによりこれを耐性細胞に変え、その耐性細胞よりA.オリーゼに対してピリチアミン耐性を付与する遺伝子(耐性遺伝子)を単離して得る。
【0017】ピリチアミンに対して鋭敏な感受性を示すアスペルギルス属糸状菌としては、表1に示すように、A.オリーゼ、A.ニガー、アスペルギルス カワチ(Aspergillus lutiensis mut.kawachi 以下 A. カワチと略記する。)、アスペルギルスアワモリ(Aspergillus awamori 以下 A. アワモリと略記する。)、アスペルギルス ソーヤ(Aspergillus sojae 以下 A. ソーヤと略記する。)、アスペルギルス シロウサミ(Aspergillus usami mut. Shiro－usami 以下 A. シロウサミと略記する。)又は A. ニドランスが挙げられる。
【0018】
【表1】

試験菌及び試験細胞	MIC (μg/ml)
A. オリーゼ	0.05
A. ニガー	1.0
A. カワチ	1.0
A. アワモリ	1.0
A. ソーヤ	1.0
A. シロウサミ	1.0
A. ニドランス	0.01

【0019】具体的には、ピリチアミンに感受性である A. オリーゼを、ニトロソグアニジン(nitrosoguanidine以下NTGと略記する。)により変異処理し、得られた耐性株についてゲノムライブラリーを作成し、そのライブラリーから優性変異であるピリチアミン耐性遺伝子(以下、ptrAと略記する。)を含有する図1の制限酵素地図で表されるDNA断片を単離する。
【0020】この発明のピリチアミン耐性遺伝子(ptrA)をコードするDNAとしては、配列表の配列番号1で表されるDNAがある。
【0021】そして、この発明のptrAをコードするDNAは、適当なベクターに組み込んで複製ベクターを作成し、宿主を形質転換すれば、宿主にピリチアミン耐性を選択マーカーとして付与することができ、ピリチアミンを用いた薬剤耐性により容易に形質転換体を選択することができる。この複製ベクターのアスペルギルス属糸状菌用ベクターとしては、pDG3、pkBY2、pSa123、pTAex3が使用できる。
【0022】また、ptrAをコードするDNAを組み込んだ複製ベクターは、大腸菌などに対して安定に保持させることが可能である。その際に、ベクターとして使用可能なものとして、PUC 系ベクター、大腸菌－アスペルギルス属糸状菌シャトルベクターなどがある。
【0023】この発明のptrAは、単核、多核のアスペルギルス属糸状菌、特に、実用麹菌にもピリチアミン耐性を付与することができるマーカー遺伝子であり、清酒、焼酎、醤油、有用物質の工業的生産等に広範に利用されているA.オリーゼの育種にきわめて有用である。
【0024】更にこの発明のptrAは、A.オリーゼ以外の麹菌にも適用が可能であり、他のアスペルギルス属糸状菌の育種、遺伝子工学的利用においても有用である。また、この発明のptrAはA.オリーゼにピリチアミン耐性を付与することができ、ptrAをコードするDNAを有するベクターは、A.オリーゼへの薬剤耐性付与ベクターとして初めて提供される物である。
【0025】
【実施例】〔実施例1〕以下の工程で、A.オリーゼ由来のピリチアミン感受性関連遺伝子(ptrA)のクローニングを行なった。
【0026】1－a) A.オリーゼのピリチアミン耐性変異株の分離ピリチアミンに0. 1ppm で感受性を示すA.オリ

ーゼ HL-1034株をチアミンを含まないCDプレート(ツアペックドックス培地、1%グルコース、2%アガー)へ植菌し、30℃で5日間培養した。着生した分生子を0.1%ツイーン80(tween 80)溶液に懸濁後、ガラスフィルター(3G3 タイプ)で濾過し、ろ液を8000rpm で5分間遠心し、分生子を回収した。

【0027】この分生子を2．4×10^7spores ／mlとなるようにNTG 溶液(4000 ppm NTG)に懸濁し、30℃で10分間、50rpm で往復振とうさせることによって突然変異を誘導した。このときの生存率は約2．2%であった。

【0028】突然変異誘導処理後、洗浄液(0．1% tween 80 、0．01M燐酸緩衝液(pH7．0))で3回分生子を洗浄し、1.0×10^7spores/plate となるようにスクリーニング用プレート(ツァペックドックス培地、1%グルコース、1ppm ピリチアミン)上に接種し、30℃、4日間培養した。これにより、ピリチアミン耐性株6株を得た。

【0029】この耐性株は1000ppm のピリチアミンに対しても耐性を示したが、オリゴマイシン、シクロヘキシミド、アンホテリシンB、クロトリマゾールに対しては親株と同様に感受性を持つことから多剤耐性ではなく、ピリチアミンに特異的な耐性であると推定された。

【0030】1－b) ピリチアミン耐性株のゲノムライブラリーの作成ピリチアミン耐性株の中で特に生育の良い株PTR-26からゲノムDNAを以下の方法により抽出して精製した。すなわち、YPD液体培地(0．5%yeast ext.,1.0% bact pepton,2% dextrin)で30℃で1晩培養した菌糸をガラスフィルター(3G1 タイプ)により集菌し、蒸留水で洗浄した。菌体を脱水し、液体窒素により凍結させ、乳鉢を用いて粉砕した。粉砕した菌体をTE溶液(10mM Tris-HCl(pH8．0)，1mM EDTA)に懸濁後、等量の溶菌溶液(2% SDS; 0．1M NaCl, 10 mM EDTA, 50 mM Tris－HCl(pH7．5))を加えた。室温で1時間静置後、3000 rpmで10分間遠心し、上清を回収した。フェノール／クロロフォルム／イソアミルアルコール(25／24／1)を等量加え、ゆっくりチューブを上下に攪拌した後、3000 rpm、5分間遠心して上層を回収した。－20℃エタノールを2．5倍量加えて、－80℃に10分間放置した後、3500 rpm、15分間遠心し、沈殿したDNAを乾燥させた。RNase 溶液(1μ/ ml RNaseGS (宝酒造社製)，TE溶液)を加え、37℃、1時間保温した。等量のフェノール／クロロホルム／イソアミルアルコールを加え、ゆっくり攪拌し、15000rpm で5分間遠心し、上層を回収した。1／10量の3M酢酸ナトリウム(pH5．2)と2．5倍量の－80℃エタノールを加え、－80℃に10分間放置後、15000rpm、15分間の遠心によりDNAを回収した。

【0031】精製したゲノムDNA10μg を制限酵素Sau3A Ⅰの0．156Uで37℃、1時間部分分解処理後、フェノール／クロロホルム／イソアミルアルコールにより除蛋白し、エタノール沈殿した。部分分解DNAを0．8%アガロース電気泳動にかけ、4～10kb領域のDNAを抽出、精製した。得られたDNAとBamHIで完全分解したpDHG25ベクター[gene，第98巻，第61頁～67頁(1991)]をDNAライゲーションキット(宝酒造社製)により連結させた後、大腸菌DH5を形質転換し、耐性株のゲノムライブラリーを作成した。

【0032】このゲノムライブラリーを含有させた大腸菌を100 ppmアンピシリンを含むLB培地(1%バクトトリプトン、0．5%バクトイーストエキス、0．5%塩化ナトリウム、PH7．2)50ml中で37℃、一晩培養後、大腸菌からプラスミドを回収し精製した。

【0033】1－c) ピリチアミン耐性遺伝子(ptrA)の発現及びクローニング上記のようにして調整したピリチアミン耐性株のゲノムライブラリー由来のプラスミドを以下の方法によりA．ニドランスFGSC A89株に形質転換した。

【0034】すなわち、A．ニドランスをチアミンを含まないCD培地等で30℃、2日間振とう培養後、菌糸をガラスフィルター(3Glタイプ)で濾過することにより回収し、滅菌水で洗浄した。十分に脱水後、菌体を10mlのプロトプラスト化溶液[20mg／mlヤタラーゼ(大関酒造社製)、0．8M NaCl 、10mM燐酸ナトリウム緩衝液、pH6．0]に懸濁した。30℃でゆっくり振とうしながら約3時間反応させた。ガラスフィルター 3G3で濾過した濾液中のプロトプラストを2000 rpm、5分間の遠心により回収し、0．8M NaClで2回洗浄した。プロトプラストを2×10^8 ／mlとなるようにSol 1(0．8M NaCl、10mM $CaCl_2$ 、10mM Tris －HCl 、pH8．0)に懸濁した後、0．2容量のSo1 2 (40%(w ／v)PEG4000 、50mM $CaC1_2$、50mM Tris-HCl 、pH8．0)を加えよく混合した。0．2mlのプロトプラスト懸濁液を分注し、10μg のゲノムライブラリー由来のプラスミドを加え、良く混合した。氷温で30分間放置後、1mlのSo12を加えよく混合した。室温で15分間放置し、8．5mlのSollを加え、よく混合後、2000rpm で5分間の遠心によりプロトプラストを回収した。0．2mlのSollを加え、2 ppmのピリチアミンを含む最小培地(ツァペックドックス培地、1%グルコース、0．8 M NaCl 、20ppb ビオチン、2%アガー)の中央にのせた後、45℃に保温した軟寒天培地(ツァペックドックス培地、1%グルコース、0．8M NaCl、20 ppbビオチン、2ppm ピリチアミン、0．5%アガー)5 mlを重層した。30℃、5～7日間培養した。

【0035】このプレート上で増殖したコロニーは、ピリチアミン耐性遺伝子を含むプラスミドを持っていると考えられ、約6個のコロニーがピリチアミン含有培地上に生じた。このコロニーを2 ppmピリチアミンを含むCD＋bi培地(ツァペックドックス培地、1%グルコース、20ppb ビオチン)へ植菌し、30℃、2日間培養し、増殖した菌体より、実施例1－b)に述べたDNA抽出精製法に従って、全DNAを回収精製した。このDNAで大腸菌DH5

を形質転換し、100 ppmアンピシリンを含むLB培地へ塗布した。生じた大腸菌コロニーからプラスミドDNAを調整した。
【0036】このプラスミドは2 kb のDNAを含んでおり、p142－13と命名した。このピリチアミン耐性遺伝子ptrAを含有するDNA断片の制限酵素地図は図1に示すとおりである。この断片をpBIISK＋ベクターへサブクローニングし、pptrAEH と命名した。その塩基配列は配列番号1に示す通りである。
【0037】ここで、ptrAと、その配列を基にPTR-26の親株であるピリチアミン感受性株HL-1034 からクローニングしたptrAの野生型遺伝子の塩基配列を比較した結果、ptrAでは配列番号1における670番目のアデニンがグアニンに置換していることが確認された。このことにより、この一塩基置換がピリチアミンに対する耐性化の原因であると推定された。
【0038】〔実施例2〕アスペルギルス属糸状菌を宿主に用いた形質転換2－a) 遊離型プラスミドを用いたアスペルギルス属糸状菌の形質転換実施例1－c)で取得したプラスミドp142－13またはpDHG25ベクターを実施例1－c)で示した形質転換方法によりA. オリーゼまたはA. ニドランスへ導入した。各濃度ピリチアミンを含む最小培地(ツァペックドックス培地、1%グルコース、0. 8M NaCl、2%アガー)の中央にのせた後、45℃に保温した各濃度のピリチアミンを含む軟寒天培地(ツァペックドックス培地、1%グルコース、0. 8 M NaCl 、0. 5%アガー)5 mlを重層した。30℃、5～7日間培養後、ピリチアミン耐性株を得た。
【0039】表2が示すようにA. オリーゼ、A. ニドランス共にp142－13による形質転換体はすべてのピリチアミン濃度において生育可能である。これに対してpDHG25ベクターによる形質転換体は、0. 1ppm のピリチアミンに対しても感受性を示していた。従って、ptrAは麹菌に対する有効な選択マーカーとして利用できることが確認された。
【0040】
【表2】

		PT 添加濃度 (ppm)			
	試験菌及び試験細胞	0	0.1	1.0	10
形質転換区分	A. オリゼー	++	++	++	++
	A. ニドランス	++	++	++	++
非形質転換区分	A. オリゼー	++	－	－	－
	A. ニドランス	++	－	－	－

【0041】2－b)染色体組込型プラスミドを用いたアスペルギルス属糸状菌の形質転換実施例1－c)で取得したプラスミドpptrAEH またはpBIISK＋を用いてA. オリーゼ、A. ニドランスまたはA. ニガーを実施例1－c)で述べた方法によって形質転換した。形質転換処理したプロトプラストを実施例2－a)で述べた各濃度のピリチアミン添加最小培地で、30℃、5～7日間培養し、ピリチアミン耐性株を得た。
【0042】
【表3】

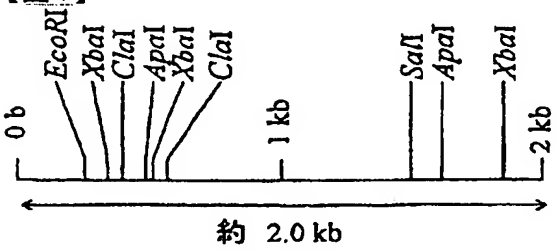
		PT 添加濃度 (ppm)			
	試験菌及び試験細胞	0	0.1	1.0	10
形質転換区分	A. オリゼー	++	++	++	++
	A. ニドランス	++	++	++	++
	A. ニガー	++	++	++	++
非形質転換区分	A. オリゼー	++	－	－	－
	A. ニドランス	++	－	－	－
	A. ニガー	++	－	－	－

【0043】表3が示すようにpptrAEH によるA. オリーゼ、A. ニドランスまたはA. ニガーの形質転換体はすべてのピリチアミン濃度において生育可能であった。これに対してpBIISK＋による形質転換体は0. 1ppm のピリチアミンに対しても感受性を示した。従って、ptrAはアスペルギルス属糸状菌に対する有効な選択マーカーとして利用できることが確認された。
【0044】
【発明の効果】本願の発明は、配列番号1で表される塩基配列のDNAからなるピリチアミン耐性マーカー遺伝子としたので、ピリチアミンに対して感受性を示す生物に耐性を付与し、遺伝子工学的育種、遺伝情報解析などの遺伝子工学的使用の際に選択マーカーとして適用できるピリチアミン耐性遺伝子を提供できるという利点がある。
【0045】また、この発明のピリチアミン耐性マーカー遺伝子は、実用麹菌などの宿主株に対して突然変異誘導された場合のような影響を与えることがなく、マーカーとしての薬剤耐性という形質を付与できる。

【0046】本願の発明により、アスペルギルス属、特に工業用麹菌の遺伝子組み換えにおいて有用なピリチアミン耐性を選択マーカーとした染色体組み換え用ベクターを導入された形質転換体、その創製方法、およびその方法で得られる形質転換体が提供される。これらは、有用蛋白質製造用形質転換体の創製、育種などに有効に利用できる。
【0047】特にA. オリーゼは、工業用麹菌として広く使用されているのみならず、遺伝子組み換えにおいても安全性の高い麹菌である。したがって、A. オリーゼ由来のピリチアミン耐性遺伝子はA. オリーゼの育種、有用蛋白質製造のための形質転換体作成のために非常に有用である。
【0048】
【配列表】
SEQUENCE LISTING<110> Hakutsuru Shuzou Kabushiki kaisha <120> Pyrithiamine resistance marker gene and transformed cell<130> KP05407-14<160> 1 <210> 1 <211> 2028<212> DNA <213> Aspergillus oryzae<400> 1 ggggatctga cagacgggca attgattacg ggatcccatt ggtaacgaaa tgtaaaagct 60aggagatcgt ccgccgatgt caggatgatt tcacttgttt cttgtccggc tcaccggtca 120 aagctaaaga ggagcaaaag gaacggatag aatcgggtgc cgctgatcta tacggtatag 180 tgcccttatc acgttgactc aacccatgct atttaactca acccctcctt ctgaacccca 240 ccatcttctt ccttttcctc tcatcccaca caattctcta tctcagattt gaattccaaa 300 agtcctcgga cgaaactgaa caagtcttcc tcccttcgat aaaccttttgg tgattggaat 360 aactgaccat cttctatagt tcccaaacca accgacaatg taaatacact cctcgattag 420 ccctctagag ggcatacgat ggaagtcatg gaatactttt ggctggactc tcacaatgat 480 caaggtatct taggtaacgt ctttggcgtg ggccggtgtt cgttcccagt catcgatgca 540 ttcacatgcc ctccctaagc tgggccctag actctaggat cctagtctag aaggacatgg 600 catcgatgga ctgggttcgt tctgagatta tacggctaaa acttgatctg gataatacca 660 gcgaaaaggg tcatgccttc tctcgttctt cctgttgatg gaatggctaa cagatgatag 720 tcattgcaac ttgaaacatg tctcctccag ctgccatcta cgaacccact gtggccgcta 780 ccggcctcaa gggtaaggtc gtggtttctg agaccgtccc cgttgaggga gcttctcaga 840 ccaagctgtt ggaccatttc ggtggcaagt gggacgagtt caagttcgcc cctatccgcg 900 aaagccaggt ctctcgtgcc atgaccagac gttactttga ggacctggac aagtacgctg 960 aaagtgacgt tgtcattgtt ggtgctggtt cctgcggtct gagcactgcg tacgtcttgg 1020ccaaggctcg tccggacctg aagattgcta tcgtcgaggc cagcgtctct cctggtcagt 1080agtccatgat ggattgcctt gcactcagct ttccggaact aacgtgcaat aggtggcggt 1140gcctggttgg gtggccaact cttttctgct atggtcatgc gccgtcccgc ggaagtcttc 1200ctgaacgagc tgggtgttcc ttacgaagag gacgcaaacc ccaactacgt tgtcgtcaag 1260cacgcctccc tgtttacctc gacactcatg tcgaaggttc tctccttccc caatgtcaag 1320ctcttcaatg ctaccgctgt tgaggacttg atcacccgtc cgaccgagaa cggcaacccc 1380cagattgctg gtgttgtcgt caactggacg ctggtcaccc ttcaccacga tgatcactcc 1440tgcatggacc ccaacactat caacgctcct gtcatcatca gtaccactgg tcacgatggg 1500ccattcggcg ccttctgtgc gaagcgcttg gtgtccatgg gcagcgtcga caagctaggt 1560ggcatgcgtg gtctcgacat gaactcggcc gaggatgcca tcgtcaagaa cacccgcgag 1620gttactaagg gcttgataat cggcggtatg gagctgtctg aaattgatgg ctttaaccgc 1680atgggcccta ccttcggtgc catggttctc agtggtgtca aggctgccga ggaggcattg 1740aaggtgttcg acgagcgtca gcgcgagtgt gctgagtaaa tgactcacta cccgaatggg 1800ttcagtgcat gaaccggatt tgtcttacgg tctttgacga taggggaatg atgattatgt 1860gatagttctg agatttgaat gaactcgtta gctcgtaatc cacatgcata tgtaaatggc 1920tgtgtcccgt atgtaacggt ggggcattct agaataatta tgtgtaacaa gaaagacagt 1980ataatacaaa caaagatgca agagcggctc atcgtcaccc catgatag 2028

図面

【図1】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